(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 March 2001 (22.03.2001)

PCT

(10) International Publication Number
WO 01/20003 A3

(51) International Patent Classification[7]: C12N 15/54, 15/11, 9/12, C12Q 1/68, A01K 67/027, G01N 33/68

(21) International Application Number: PCT/EP00/09896

(22) International Filing Date:
11 September 2000 (11.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
99402236.6 10 September 1999 (10.09.1999) EP
00401388.4 18 May 2000 (18.05.2000) EP

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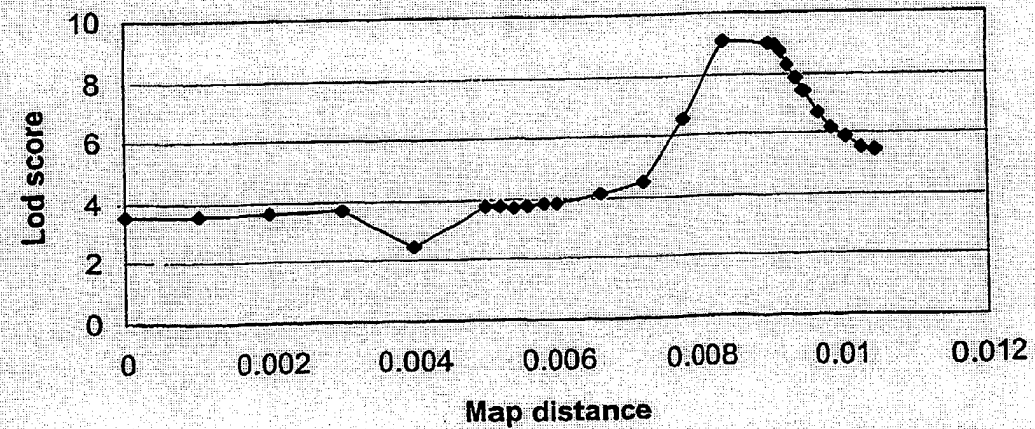
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

[Continued on next page]

(54) Title: VARIANTS OF THE GAMMA CHAIN OF AMPK, DNA SEQUENCES ENCODING THE SAME, AND USES THEREOF



(57) Abstract: The invention concerns variants of the gamma chain of vertebrate AMP-activated kinase (AMPK), as well as nucleic acid sequences encoding said variants and use thereof for the diagnosis or treatment of dysfunction of energy metabolisms.

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— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(88) Date of publication of the international search report:
17 May 2001

1998, *supra*); however, whether glycogen synthase is a physiological target of AMPK *in vivo* remained unclear.

Several isoforms of the three different AMPK subunits are present in mammals. In humans, *PRKAA1* on human chromosome (HSA) 5p12 and *PRKAA2* on HSA1p31 respectively encode isoforms α1 and α2 of the α subunit, *PRKAB1* on HSA12q24.1 and *PRKAB2* (not yet mapped) respectively encode isoforms β1 and β2 of the β subunit, and *PRKAG1* on HSA12q13.1 and *PRKAG2* on HSA7q35-q36 respectively encode isoforms γ1 and γ2 of the γ subunit (OMIM database, http://www.ncbi.nlm.nih.gov/omim/, July 1999). HARDIE et al., [1998, *supra*] also mention the existence of a third isoform (γ3) of the γ subunit of AMPK but do not provide any information about it. Analysis of the sequences of these γ subunits shows that they are essentially composed of four cystathione β synthase (CBS) domains whose function is unknown. No phenotypic effect resulting from a mutation in either of the AMPK subunits has yet been documented.

On the other hand, it has been observed that most Hampshire pigs have a high intramuscular glycogen concentration. In these pigs, glycogenolysis which occurs after slaughtering leads to an important decrease of the pH, resulting in acid meat having a reduced water-holding capacity and giving a reduced yield of cured cooked ham.

The locus (named *RN*) associated with high muscular content of glycogen was first identified by family segregation analysis of phenotypic data from Hampshire pigs (LE ROY *et al.*, Genet. Res., 55, 33-40, 1990). A fully dominant allele, *RN*, correlated with high glycogen content occurs at a high frequency in most Hampshire populations while pigs from other breeds are assumed to be homozygous for the normal, recessive rn^+ allele. Subsequent studies showed that *RN* carriers have a large increase (about 70%) of glycogen in skeletal muscle

but not in liver (MONIN *et al.*, in *38th ICoMST*, Clermont-Ferrand, FRANCE, 1992).

The large difference in glycogen content between *RN⁻* and rn⁺ pigs leads to marked differences in meat quality and technological yield (ENFÄLT *et al.*, J. Anim. Sci., 75, 2924-2935, 1997). The *RN⁻* allele is therefore of considerable economical significance in the pig industry and most breeding companies would like to reduce or eliminate this dominant mutation.

The RN phenotype can be determined by measuring the glycolytic potential in muscle biopsies from live animals, or after slaughter (MONIN *et al.*, Meat Science, 13, 49-63, 1985). However, this method has severe limitations for application in practical breeding programs. The accuracy of the test is not 100%: as there is some overlap in the phenotypic distribution of *RN⁻* and rn⁺, the test is not able to distinguish *RN⁻/RN⁻* homozygotes and *RN⁻/rn⁺* heterozygotes. Further, the sampling of muscle biopsies on live animals is invasive and costly.

Thus, there is a strong need for the development of a simple diagnostic DNA test for the *RN* locus. Moreover, the dramatic phenotypic effect of the *RN* gene in pigs implies that this gene has an important role in the regulation of carbohydrate metabolism in skeletal muscle in other vertebrates, in particular mammals.

Skeletal muscle and liver are the two major reservoirs of glycogen in mammals and the observation of an increased muscular glycogen while liver glycogen is normal suggests that the RN⁻ phenotype maybe due to a mutation in a gene expressed in muscle but not in liver. The inventors have previously reported that the *RN* gene is located on pig chromosome 15 (MILAN *et al.*, Mamm. Genome, 7, 47-51, 1996; MARIANI *et al.*, Mamm. Genome, 7, 52-54, 1996; LOOFT *et al.*, Genetics Selection Evolution, 28, 437-442, 1996). They have now discovered that the *RN⁻*

allele is associated with a non-conservative mutation in a gene encoding a new muscle-specific isoform of the AMP-activated protein kinase (AMPK) γ chain.

The various aspects of the present invention are based upon the discovery and characterisation of this mutation and the identification and isolation of the mutant gene.

According to the invention it is shown that a mutation in a γ chain of AMPK results in an altered regulation of carbohydrate metabolism, demonstrating that AMPK is an essential component of said metabolism. It is also provided a nucleic acid sequence encoding a muscle-specific isoform of the γ chain of AMPK. Thus it is provided means to regulate carbohydrate metabolism, more specifically to detect and/or correct potential or actual dysfunctions of the regulation of carbohydrate metabolism, in particular in skeletal muscle.

The invention provides a polypeptide comprising an amino acid sequence having at least 70% identity or at least 85% similarity, preferably 80% identity or at least 90% similarity, more preferably at least 90% identity or at least 95% similarity, and still more preferably at least 95% identity or at least 99% similarity, with the polypeptide SEQ ID NO: 2. The invention also provides an isolated nucleic acid sequence encoding said polypeptide, as well as the complement of said nucleic acid sequence.

Said polypeptide represents a new muscle-specific isoform of the γ chain of AMPK, and will also be hereinafter referred as Prkag3; the gene encoding said polypeptide will also be hereinafter referred as *PRKAG3*.

According to a preferred embodiment of the invention, said polypeptide comprises an amino acid sequence having at least 75% identity, preferably at least 80% identity with the polypeptide SEQ ID NO: 28.

"Identity" of a sequence with a reference sequence refers to the percent of residues that are the same when the two sequences are aligned for maximum correspondence between residues positions. A polypeptide having an amino acid sequence having at least X% identity with a reference sequence is defined herein as a polypeptide whose sequence may include up to 100-X amino acid alterations per each 100 amino acids of the reference amino acid sequence. Amino acids alterations include deletion, substitution or insertion of consecutive or scattered amino acid residues in the reference sequence.

"Similarity" of a sequence with a reference sequence refers to the percent of residues that are the same or only differ by conservative amino acid substitutions when the two sequences are aligned for maximum correspondence between residues positions. A conservative amino acid substitution is defined as the substitution of an amino acid residue for another amino acid residue with similar chemical properties (e.g. size, charge or polarity), which generally does not change the functional properties of the protein. A polypeptide having an amino acid sequence having at least X% similarity with a reference sequence is defined herein as a polypeptide whose sequence may include up to (100-X) non-conservative amino acid alterations per each 100 amino acids of the reference amino acid sequence. Non-conservative amino acids alterations include deletion, insertion, or non-conservative substitution of consecutive or scattered amino acid residues in the reference sequence.

For instance:

* searching the "GenBank nr" database using BLASTp (ALTSCHUL *et al.*, Nucleic Acids Res., 25, 3389-3402, 1997) with default settings and the whole sequence

SEQ ID NO: 2 as a query, the higher percents of identity or similarity with SEQ ID NO: 2 were found for:

- γ1 subunit of human AMPK: 65% identity or 82% similarity (score: 399);
- γ1 subunit of rat AMPK: 65% identity or 82% similarity (score: 399);
- γ1 subunit of murine AMPK: 64% identity or 80% similarity (score: 390);
- γ subunit of Drosophila AMPK: 53% identity or 75% similarity (score: 332);
- Yeast Snf4: 33% identity or 56% similarity (score: 173);

* searching the "GenBank nr" database using BLASTp with default settings and the whole sequence SEQ ID NO: 28 as a query, the higher percents of identity or similarity were found for:

- γ1 subunit of human AMPK: 64% identity or 80% similarity (score: 403);
- γ2 subunit of human AMPK: 62% identity or 83% similarity (score: 425);
- γ1 subunit of rat AMPK: 61% identity or 77% similarity (score: 404);
- γ1 subunit of murine AMPK: 63% identity or 79% similarity (score: 394);
- γ subunit of Drosophila AMPK: 52% identity or 76% similarity (score: 340).

Polypeptides of the invention include for instance any polypeptide (whether natural, synthetic, semi-synthetic, or recombinant) from any vertebrate species, more specifically from birds, such as poultry, or mammals, including bovine, ovine, porcine, murine, equine, and human, and comprising, or consisting of, the amino acid sequence of either:

- a functional Prkag3; or
- a functionally altered mutant of Prkag3.

"Functional" refers to a protein having a normal biological activity. Such a protein may comprise silent mutations inducing no substantial change in its activity, and having no noticeable phenotypic effects. Non-limitative examples of functional Prkag3 are:

- a porcine Prkag3 comprising at least the sequence represented in the enclosed sequence listing under SEQ ID NO: 2; this includes, for instance the polypeptide SEQ ID NO: 28;
- a human Prkag3 comprising at least the sequence represented in the enclosed sequence listing under SEQ ID NO: 4; this includes for instance the polypeptide SEQ ID NO: 30.

The invention also includes splice variants of Prkag3: for instance, the nucleotide sequence SEQ ID NO: 27, and the corresponding amino-acid sequence SEQ ID NO: 28 on one hand, and the nucleotide sequence SEQ ID NO: 31 and the corresponding amino-acid sequence SEQ ID NO: 32 on the other hand represent two different splice variants of porcine Prkag3.

A "functionally altered mutant" of a protein comprises one or several mutations inducing a change in its activity. Such mutations include in particular deletions, insertions, or substitutions of amino acid residues in a domain essential for the biological activity of said protein. They may result for instance in a partial or total loss of activity, or conversely in an increase of activity, or in an impairment of the response to regulatory effectors. Deletions, insertions, or non-conservative substitutions are more likely to result in a critical effect on the biological activity; however conservative substitutions may also induce a noticeable effect, if they occur at an important position of an active site of the protein.

Non-limitative examples of functionally altered mutants of Prkag3 are:

- the R41Q variant resulting from the non-conservative substitution of an arginine residue in position 41 of SEQ ID NO: 2 or SEQ ID NO: 4 by a glutamine residue (this substitution results in an important increase of the glycogen content, inducing an increased glycolytic potential of the skeletal muscle);
- the V40I variant resulting from the substitution of a valine residue in position 40 of SEQ ID NO: 2 or SEQ ID NO: 4 by an isoleucine residue (this substitution results in a decrease of the glycogen content and thus of the glycolytic potential of the skeletal muscle).

These substitutions occur inside a portion of the first CBS domain that is highly conserved between Prkag3 and the previously known isoforms of the γ subunit of AMPK.

Residue numbers for Prkag3 refer to the amino acid numbering of SEQ ID NO: 2 or SEQ ID NO: 4. Alignment of human and porcine Prkag3 sequences with previously known γ1 and γ2 isoforms is shown in Figure 3.

The invention also provides mutants of Prkag3 which may for instance be obtained by deletion of part of a Prkag3 polypeptide. Said mutants are generally functionally altered. They may have an identity with the overall Prkag3 sequence lower than 70%. However, the identity of the non-deleted sequences of said mutants, when aligned with the corresponding Prkag3 sequences and more specifically with the corresponding sequences from SEQU ID NO: 2, should remain higher than 70%. Said mutants may for instance result from the expression of nucleic acid sequences obtained by deletion or insertion of a nucleic acid segment, or by a punctual mutation introducing a nonsense codon, in a nucleic acid sequence encoding a functional Prkag3.

The invention also provides a functionally altered mutant of a γ subunit of AMPK, wherein said mutant comprises at least one mutation responsible for said functional alteration located within the first CBS domain, and preferably within the region thereof aligned with the region spanning from residue 30 to residue 50 of SEQ ID NO:2 or SEQ ID NO:4. Said mutation may result from the insertion, deletion, and/or substitution of one amino-acid or of several amino-acids, adjacent or not. More preferably the mutation is located within the region aligned with the region spanning from residue 35 to residue 45 of SEQ ID NO:2 or SEQ ID NO:4, for instance within the region spanning from residue 65 to residue 75 of the γ1 isoform.

According to a particular embodiment, said mutation is a non-conservative substitution, preferably a R→Q substitution. According to another particular embodiment, said mutation is a conservative substitution, preferably a V→I substitution.

Advantageously, the mutation is located at a residue corresponding to residue 41 of SEQ ID NO:2 or SEQ ID NO:4, for instance in the case of the γ1 isoform, at residue 70, or at a residue corresponding to residue 40 of SEQ ID NO:2 or SEQ ID NO:4, for instance in the case of the γ1 isoform, at residue 69.

The invention also provides a heterotrimeric AMPK wherein the γ subunit consists of a polypeptide of the invention.

The invention also provides isolated nucleic acid sequences encoding any of the above-defined functional or functionally altered Prkag3 or functionally altered mutants of a γ subunit of AMPK, and nucleic acid sequences complementary of any one of these nucleic acid sequences.

This includes particularly any isolated nucleic acid having the sequence of any of the naturally

occurring alleles of a *PRKAG3* gene, as well as any isolated nucleic acid having the sequence of an artificial mutant of a *PRKAG3* gene, provided that said nucleic acid does not consist of the EST GENBANK AA178898.

This also includes any isolated nucleic acid having the sequence of a natural or artificial mutant of a *PRKAG1* or a *PRKAG2* gene, wherein said mutant encodes a functionally altered γ1 or γ2 subunit of AMPK as defined above.

Nucleic acids of the invention may be obtained by the well-known methods of recombinant DNA technology and/or of chemical DNA synthesis. These methods also allow to introduce the desired mutations in a naturally occurring DNA sequence.

Examples of nucleic acids encoding naturally occurring alleles of a *PRKAG3* gene are represented by SEQ ID NO: 1, which encodes a naturally occurring allele of the porcine gene and SEQ ID NO: 3, which encodes a naturally occurring allele of the human gene. These sequences may be used to generate probes allowing the isolation of *PRKAG3* from other species or of other allelic forms of *PRKAG3* from a same species, by screening a library of genomic DNA or of cDNA.

The invention also includes genomic DNA sequences from any vertebrate species, more specifically from birds, such as poultry, or mammals, including in particular bovine, ovine, porcine, murine, equine, and human, comprising at least a portion of a nucleic acid sequence encoding a polypeptide of the invention, preferably a portion of a *PRKAG3* gene, and up to 500 kb, preferably up to 100 kb of a 3′ and/or of a 5′ adjacent genomic sequence.

Such genomic DNA sequences may be obtained by methods known in the art, for instance by extension of a nucleic acid sequence encoding a polypeptide of the

invention, employing a method such as restriction-site PCR (SARKAR *et al.*, PCR Methods Applic., 2, 318-322, 1993), inverse PCR (TRIGLIA *et al.*, Nucleic Acids Res., 16, 8186, 1988) using divergent primers based on a Prkag3 coding region, capture PCR (LAGERSTROM *et al.*, PCR Methods Applic., 1, 111-119, 1991), or the like.

The invention also includes specific fragments of a nucleic acid sequence encoding a polypeptide of the invention, or of a genomic DNA sequence of the invention as well as nucleic acid fragments specifically hybridising therewith. Preferably these fragments are at least 15bp long, more preferably at least 20bp long.

"Specific fragments" refers to nucleic acid fragments having a sequence that is found only in the nucleic acids sequences encoding a polypeptide of the invention, and is not found in nucleic acids sequences encoding related polypeptides of the prior art. This excludes the nucleic acid fragments that consist of a sequence shared with one of the known *PRKAG1* or *PRKAG2* genes.

"Specifically hybridising fragments" refers to nucleic acid fragments which can hybridise, under stringent conditions, only with nucleic acid sequences encoding a polypeptide of the invention, without hybridising with nucleic acid sequences encoding related polypeptides of the prior art. This excludes the nucleic acid fragments that consist of the complement of a sequence shared with one of the known *PRKAG1* or *PRKAG2* genes.

Nucleic acid fragments that consist of the EST GENBANK AA178898 or the EST GENBANK W94830 or the complements thereof are also excluded.

Said specific or specifically hybridising nucleic acid fragments may for example be used as primers or probes for detecting and/or amplifying a nucleic acid sequence encoding a polypeptide of the invention. The

invention encompasses set of primers comprising at least one primer consisting of a specific or specifically hybridising nucleic acid fragment as defined above.

The invention also provides recombinant vectors comprising a nucleic acid sequence encoding a polypeptide of the invention. Vectors of the invention are preferably expression vectors, wherein a sequence encoding a polypeptide of the invention is placed under control of appropriate transcriptional and translational control elements. These vectors may be obtained and introduced in a host cell by the well-known recombinant DNA and genetic engineering techniques.

The invention also comprises a prokaryotic or eukaryotic host cell transformed by a vector of the invention, preferably an expression vector.

A polypeptide of the invention may be obtained by culturing the host cell containing an expression vector comprising a nucleic acid sequence encoding said polypeptide, under conditions suitable for the expression of the polypeptide, and recovering the polypeptide from the host cell culture.

A heterotrimeric AMPK wherein the γ subunit consists of a polypeptide of the invention may be obtained by expressing, together or separately, a nucleic acid sequence encoding a polypeptide of the invention, a nucleic acid sequence encoding an α subunit, and a nucleic acid sequence encoding a β subunit, and reconstituting the heterotrimer.

The polypeptides thus obtained, or immunogenic fragments thereof may be used to prepare antibodies, employing methods well known in the art. Antibodies directed against the whole Prkag3 polypeptide and able to recognise any variant thereof may thus be obtained. Antibodies directed against a specific epitope of a particular variant (functional or not) of Prkag3 or antibodies directed against a specific epitope of a

functionally altered mutant having a mutation in the first CBS domain of a γ subunit of AMPK, and able to recognise said variant or functionally altered mutant may also be obtained.

As shown herein, mutations in a γ subunit of AMPK, and particularly mutations in the first CBS domain of a γ subunit of AMPK are likely to cause disorders in the energy metabolism (e.g. diabetes, obesity) in vertebrates, including humans. Further, mutations in the first CBS domain or other parts of the *PRKAG3* gene are likely to cause disorders in the muscular metabolism leading to diseases such as myopathy, diabetes and cardiovascular diseases.

The present invention provides means for detecting and correcting said disorders.

More specifically, the present invention is directed to methods that utilise the nucleic acid sequences and/or polypeptidic sequences of the invention for the diagnostic evaluation, genetic testing and prognosis of a metabolic disorder.

For example, the invention provides methods for diagnosing of metabolic disorders, more specifically carbohydrate metabolism disorders, and preferably disorders correlated with an altered, in particular an excessive, glycogen accumulation in the cells, resulting from a mutation in a gene encoding a γ subunit of AMPK, wherein said methods comprise detecting and/or measuring the expression of a functionally altered *PRKAG3* gene, or of a functionally altered mutant of a γ subunit of AMPK having a mutation within the first CBS domain in a nucleic acid sample obtained from a vertebrate, or detecting a mutation in the *PRKAG3* gene or in a sequence encoding the first CBS domain of a γ subunit of AMPK in the genome of a vertebrate suspected of having such a disorder.

According to a preferred embodiment of the invention, the disorder is correlated with an altered, in particular an excessive, glycogen accumulation in the muscular cells and results from the expression of a functionally altered *PRKAG3* gene.

The expression of a functionally altered Prkag3, or of a functionally altered mutant of a γ subunit of AMPK having a mutation within the first CBS domain may be detected or measured using either polyclonal or monoclonal antibodies specific for the functionally altered polypeptides of the invention, as defined above. Appropriate methods are known in the art. They include for instance enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS).

The nucleotide sequences of the invention may be used for detecting mutations in the *PRKAG3* gene or in a sequence encoding the first CBS domain of a γ subunit of AMPK, by detection of differences in gene sequences or in adjacent sequences between normal, carrier, or affected individuals.

The invention provides a process for detecting a mutation in the *PRKAG3* gene or in a sequence encoding the first CBS domain of a γ subunit of AMPK wherein said process comprises:

- obtaining a nucleic acid sample from a vertebrate;
- checking the presence in said nucleic acid sample of a nucleic acid sequence encoding a mutant Prkag3, or a mutant of a γ subunit of AMPK having a mutation within the first CBS domain, as defined above.

According to a preferred embodiment of the invention there is provided a method for detecting a nucleic acid sequence comprising a mutation in the *PRKAG3* gene or in a sequence encoding the first CBS domain of a γ subunit of AMPK wherein said process comprises:

- obtaining a nucleic acid sample from a vertebrate;

- contacting said nucleic acid sample with a nucleic acid probe obtained from a nucleic acid of the invention and spanning said mutation, under conditions of specific hybridisation between said probe and the mutant sequence to be detected;
- detecting the hybridisation complex.

Preferably, the process of the invention further comprises, prior to hybridisation, PCR amplification from the nucleic acid sample, of a sequence comprising at least the portion of the *PRKAG3* sequence or of the sequence encoding the first CBS domain of the γ subunit of AMPK wherein the mutation is to be detected.

Methods allowing the specific hybridisation of a probe only with a perfectly matching complementary sequence, and useful for the detection of punctual mutations are known in the art. They include for instance Allele Specific PCR (GIBBS, Nucleic Acid Res., 17, 2427-2448, 1989), Allele Specific Oligonucleotide Screening (SAIKI *et al.*, Nature, 324, 163-166, 1986), and the like.

A mutation in the *PRKAG3* gene may also be detected through detection of polymorphic markers closely linked to said mutation.

The invention also provides means for identifying said polymorphic markers, and more specifically polymorphic markers comprised within a genomic DNA sequence comprising at least a portion of a *PRKAG3* gene, and up to 500 kb, preferably 300 kb, more preferably up to 100 kb of a 3' and/or of a 5' adjacent sequence.

Said polymorphic markers may be obtained for instance, by screening a genomic DNA library from a vertebrate with a probe specific for the *PRKAG3* gene, in order to select clones comprising said nucleic acid sequence and flanking chromosomal sequences, and identifying a polymorphic marker in said flanking chromosomal sequences. The allele(s) of a polymorphic

marker associated with a given mutant allele of the *PRKAG3* gene may also easily be identified by use of a genomic DNA library from an individual wherein the presence of said mutant allele has previously been detected by hybridisation with a nucleic acid probe of the invention.

Polymorphic markers include for instance, single nucleotide polymorphisms (SNP), microsatellites, insertion/deletion polymorphism and restriction fragment length polymorphism (RFLP). These polymorphic markers may be identified by comparison of sequences flanking the *PRKAG3* gene obtained from several individuals. Microsatellites may also be identified by hybridisation with a nucleic acid probe specific of known microsatellite motifs.

Once a polymorphic marker has been identified, a DNA segment spanning the polymorphic locus may be sequenced and a set of primers allowing amplification of said DNA segment may be designed.

The invention also encompasses said DNA primers.

Detection of a mutation in the *PRKAG3* gene may be performed by obtaining a sample of genomic DNA from a vertebrate, amplifying a segment of said DNA spanning a polymorphic marker by polymerase chain reaction using a set of primers of the invention, and detecting in said amplified DNA the presence of an allele of said polymorphic marker associated with said mutation.

By way of example, polymorphic markers which may be obtained according to the invention, and DNA primers allowing the detection of polymorphic markers closely linked to the *RN*$^-$ allele of porcine *PRKAG3* gene are listed in Table 1 hereinafter.

According to a preferred embodiment of the invention, the vertebrate is a mammal, preferably a farm animal and more preferably a porcine, and the mutation to

be detected produces a functionally altered Prkag3. The detection of said mutation allows to predict whether said mammal or the progeny thereof is likely to have an intramuscular glycogen concentration higher or lower than the average. An example of such a mutation produces a functionally altered Prkag3 having a R41Q substitution, and resulting in an increased glycogen content in the skeletal muscle.

Another example of such a mutation produces a functionally altered Prkag3 having a V40I substitution, and resulting in a decreased glycogen content in the skeletal muscle. In farm animals having such a mutation, glycogenolysis which occurs after slaughtering is less important than in normal animals, resulting in a higher pH and in a potential better quality of the meat.

The present invention also includes kits for the practice of the methods of the invention. The kits comprise any container which contains at least one specific fragment of a nucleic acid sequence of the invention, or at least one nucleic acid fragment able to specifically hybridise with a nucleic acid sequence of the invention. Said nucleic acid fragment may be labelled. The kits may also comprise a set of primers of the invention. They may be used in conjunction with commercially available amplification kits. They may also include positive or negative control reactions or markers, molecular weight size markers for gel electrophoresis, and the like.

Other kits of the invention may include antibodies of the invention, optionally labelled, as well as the appropriate reagents for detecting an antigen-antibody reaction. They may also include positive or negative control reactions or markers.

The invention further provides means for modulating the expression of vertebrate genes encoding a γ subunit of AMPK, and more specifically of the *PRKAG3* gene

and/or the synthesis or activity of the products of said genes.

A purified AMPK heterotrimer comprising wild-type or mutant Prkag3 subunit, or a functionally altered mutant γ subunit having a mutation in the first CBS domain, may be used for screening *in vitro* compounds able to modulate AMPK activity, or to restore altered AMPK activity. This may be done, for instance, by:

- measuring the binding of the compound to said heterotrimer, using for example high-throughput screening methods; or,
- measuring changes in AMPK kinase activity, using for example high-throughput screening methods.

High throughput screening methods are disclosed, for instance, in "High throughput screening: The Discovery of Bioactive Substances", J.P. DEVLIN (Ed), MARCEL DEKKER Inc., New York (1997).

Nucleic acids of the invention may be used for therapeutic purposes. For instance, complementary molecules or fragments thereof (antisense oligonucleotides) may be used to modulate AMPK activity, more specifically in muscular tissue.

Also, a nucleic acid sequence encoding a functional Prkag3 may be used for restoring a normal AMPK function.

Transformed cells or animal tissues expressing a wild-type or mutant Prkag3, or a functionally altered mutant of a γ subunit of AMPK as defined above, or expressing an AMPK comprising said mutant Prkag3, or said functionally altered mutant of a γ subunit of AMPK, may be used as *in vitro* model for elucidating the mechanism of AMPK activity or for screening compounds able to modulate the expression of AMPK.

The screening may be performed by adding the compound to be tested to the culture medium of said cells or said tissues, and measuring alterations in energy

metabolism in said cells or said tissues using methods such as measurements of glucose concentrations (levels), glucose uptake, or changes of the ATP/AMP ratio, glycogen or lipid/protein content.

The invention provides animals transformed with a nucleic acid sequence of the invention.

In one embodiment, said animals are transgenic animals having at least a transgene comprising a nucleic acid of the invention.

In another embodiment, said animals are knockout animals. "Knockout animals" refers to animals whose native or endogenous *PRKAG3* alleles have been inactivated and which produce no functional Prkag3 of their own.

In light of the disclosure of the invention of DNA sequences encoding a wild-type or mutant Prkag3, or a functionally altered mutant of a γ subunit of AMPK, transgenic animals as well as knockout animals may be produced in accordance with techniques known in the art, for instance by means of *in vivo* homologous recombination.

Suitable methods for the preparation of transgenic or knock-out animals are for instance disclosed in: *Manipulating the Mouse Embryo*, 2nd Ed., by HOGAN *et al.*, Cold Spring Harbor Laboratory Press, 1994; *Transgenic Animal Technology*, edited by C. PINKERT, Academic Press Inc., 1994; *Gene Targeting: A Practical Approach*, edited by A.L. JOYNER, Oxford University Press, 1995; *Strategies in Transgenic Animal Science*, edited y G.M. MONASTERSKY and J.M. ROBL, ASM Press, 1995; *Mouse Genetics: Concepts and Applications*, by Lee M. SILVER, Oxford University Press, 1995.

These animals may be used as models for metabolic diseases and disorders, more specifically for diseases and disorders of glycogen metabolism in muscle. For instance they may be used for screening test

molecules. Transgenic animals may thus be used for screening compounds able to modulate AMPK activity. Knockout animals of the invention may be used, in particular, for screening compounds able to modulate energy metabolism, more specifically carbohydrate metabolism, in the absence of functional Prkag3.

The screening may be performed by administering the compound to be tested to the animal, and measuring alterations in energy metabolism in said animal using methods such as glucose tolerance tests, measurements of insulin levels in blood, changes of the ATP/AMP ratio, glycogen or lipid/protein content in tissues and cells.

Transgenic or knock-out farm animals with modified meat characteristics or modified energy metabolism may also be obtained.

The present invention will be further illustrated by the additional description which follows, which refers to examples of obtention and use of nucleic acids of the invention. It should be understood however that these examples are given only by way of illustration of the invention and do not constitute in any way a limitation thereof.

EXAMPLE 1: ISOLATING THE *PRKAG3* GENE

We have screened a porcine Bacterial Artificial Chromosome (BAC) library (ROGEL-GAILLARD *et al.*, Cytogenet and Cell Genet, 851, 273-278, 1999) and constructed a contig of overlapping BAC clones across the region of pig chromosome 15 harbouring the *RN* gene. These BAC clones were in turn used to develop new genetic markers in the form of single nucleotide polymorphisms (SNPs) or microsatellites (MS) as described in Table 1 below.

Table 1

	Name of marker	BAC clone	Primer sequences	Size of PCR product (bp)	Marker type[a]	Alleles[b]
1	H3	115B9, 156E6, 361B4, 90A9	F; 5'-GGAATTTCAAGTCAGCCAAC-3' (SEQ ID NO: 5) R; 5'-CTTCAAAAGACCGTGCTACT-3' (SEQ ID NO: 6)	114 –138	MS	114, 126, 128, 132*, 134*, 136, 138
2	MS982H1	982H11	F; 5'-CTGGGAACCTCTATATGCTG-3' (SEQ ID NO: 7) R; 5'-TAGGGAAATACAAATCACAG-3' (SEQ ID NO: 8)	114 - 157	MS	114, 140, 142*, 144, 146, 150, 158
3	MS479L3	479L3, 297D7, 852B5, 153B5	F; 5'-CTCCAGCTCACAGGATGACA-3' (SEQ ID NO: 9) R; 5'-GTTTCTGCAGCTTTAGCATCTATTCC-3' (SEQ ID NO: 10)	150 - 164	MS	150*, 160, 162, 164
4	MS997M35	997F12	F; 5'-GAAGTATCCTGGGCTTCTGA-3' (SEQ ID NO: 11) R; 5'-GTTTCTCCAGGTTTCCAGACATCCAC-3' (SEQ ID NO: 12)	138 - 160	MS	138, 144, 152, 154, 160*
5	MS482H65	482E7	F; 5'-GCTTCTGTCTGCCCCTACTT-3' (SEQ ID NO: 13) R; 5'-GTTTCTAAGTTCTACTGTAAGACACC-3' (SEQ ID NO: 14)	78 - 90	MS	78, 80, 88*, 90
6	MS337H2	808G10, 947E5, 337G11	F; 5'-CCAAGCTGTGGTGGCTGAAT-3' (SEQ ID NO: 15) R; 5'-CAGCACAGCAGTGCCACCTA-3' (SEQ ID NO: 16)	145 - 165	MS	145, 149, 155, 161*, 165*
7	MS127B1	127G6, 134C9	F; 5'-CAAACTCTTCTAGGCGTGT-3' (SEQ ID NO: 17) R; 5'-GTTTCTGGAACTTCCATATGCCATGG-3' (SEQ ID NO: 18)	94 - 108	MS	94, 100, 108*, 114
8	CMKAR2	128A3, 337G11, 808G10, 947E7, 1110H12	F; 5'-AGGGTGGATGGTAGGCTTCA-3' (SEQ ID NO: 19) R; 5'-GTCTCGCTCCTGAAGGAAGT-3' (SEQ ID NO: 20)	208	SNP	112A*, 112T; 158A*, 158G 176A*, 176G
9	127G63	127G6, 134C9, 170D7, 1030A5,	F; 5'-AGTCACGTGGCCATGCTATC-3' (SEQ ID NO: 21) R; 5'-CTCAACTGGATTGAGTCAGT-3' (SEQ ID NO: 22)	409	SNP	234A*, 234C
10	VIL1	1088F2	F; 5'-TTGGCGCAACTGTTATTTCT-3' (SEQ ID NO: 23) R; 5'-AGGCAAAGGAAGAGCACAG-3' (SEQ ID NO: 24)	270	SNP	90T, 90G, 120A, 120G, 166C, 166T
11	NRAMP1	315F7, 530A6, 651C12, 1088F2, 1095H3	F; 5'-AGCCGTGGGCATCGTTGG-3' (SEQ ID NO: 25) R; 5'-AGAAGGAGACAGACAGGGCGA-3' (SEQ ID NO:26)	1300	RFLP (*StyI*)	1: 100+1200 bp 2:100+200+1000 bp

[a]MS=microsatellite; SNP=single nucleotide polymorphism.
[b]Microsatellite alleles are designated according to the length of the amplified fragment while SNPs are denoted according to the polymorphic nucleotide. Alleles associated with the *RN* allele are marked with an asterisk.

The new markers were used together with some previously described markers to construct a high-resolution linkage map. Standard linkage analysis using pedigree data comprising about 1,000 informative meioses for segregation at the *RN* locus made it possible to exclude *RN* from the region proximal to *MS479L3* and distal to microsatellite *Sw936*. Linkage Disequilibrium (LD) analysis was done with the same markers and a random sample of 68 breeding boars from the Swedish Hampshire population, scored for the RN phenotype by measuring glycogen content in muscle. The results of LD analysis using the DISMULT program (TERWILLIGER, Am. J. Hum. Genet., 56, 777-787, 1995) are shown in Figure 1. They reveal a sharp LD peak around the markers MS127B1 and SNP127G63. These markers appeared to show complete linkage disequilibrium with the *RN* allele, i.e. *RN* was associated with a single allele at these two loci. The most simple interpretation of this finding is that the *RN* mutation arose on a chromosome carrying these alleles and that the two markers are so closely linked to the *RN* locus that the recombination frequency is close to 0%. The two markers are both present on the overlapping BAC clones 127G6 and 134C9 suggesting that the *RN* gene may reside on the same clone or one of the neighbouring clones.

A shot-gun library of the BAC clone 127G6 was constructed and more than 1,000 sequence reads were collected giving about 500,000 base pair random DNA sequence from the clone. The data were analysed and sequence contigs constructed with the PHRED, PHRAP and CONSED software package (University of Washington Genome Center, http://bozeman.mbt.washington.edu). The sequence data were masked for repeats using the REPEATMASKER software (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker) and BLAST searches were carried out using the NCBI web site (http://www.ncbi.nlm.nih.gov).

Three convincing matches to coding sequences were obtained. Two of these were against human cDNA sequences/genes, KIAA0173 described as being similar to pig tubulin-tyrosine ligase and located on HSA2q (UniGene cluster Hs.169910, http://www.ncbi.nlm.nih.gov/UniGene/) and CYP27A1 located on HSA2q33-ter (UniGene cluster Hs. 82568). The results strongly suggested that the pig coding sequences are orthologous to these human genes as it is well established that the *RN* region is homologous to HSA2q33-36 (ROBIC *et al.*, Mamm. Genome, 10, 565-568, 1999). However, none of these sequences appeared as plausible candidate genes for *RN*. The third coding sequence identified in BAC 127G6 showed highly significant sequence similarity to various AMP-activated protein kinase γ sequences including the yeast *SNF4* sequence. The cDNA sequence of this gene was determined by RT-PCR and RACE analysis using muscle mRNA from an rn^+/rn^+ homozygote. This sequence is shown in Figure 2 and in the enclosed sequence listing under SEQ ID NO: 1.

Legend of Figure 2:
5' UTR: 5' untranslated region
3' UTR: 3' untranslated region
CDS: coding sequence
***: stop codon
'-': identity to master sequence
'.': alignment gap

The frame of translation was determined on the basis of homology to other members in the protein family and assuming that the first methionine codon in frame is the start codon. The polypeptidic sequence deduced on this basis is shown in the enclosed sequence listing under SEQ ID NO: 2.

The complete nucleotidic sequence of pig PRKAG3 cDNA is shown in the enclosed sequence listing under SEQ ID NO: 27 and the complete polypeptidic

sequence is shown in the enclosed sequence listing under SEQ ID NO: 28 and in Figure 3.

Figure 3 shows an amino acid alignment constructed with the CLUSTAL W program (THOMPSON *et al.*, Nucleic Acids Research, 22, 4673-4680, 1994) with representative AMPK γ sequences in the nucleotide databases.

Legend of Figure 3:

Sequences used:

HumG1: Genbank U42412

MusG1: Genbank AF036535

HumG2: Human PRKAG2 (Genbank AJ249976)

PigG3: pig PRKAG3 (this study)

HumG3: human PRKAG3 (this study)

Dros: *Drosophila* (Genbank AF094764)

SNF4 (yeast): Genbank M30470

Both the PRKAG2 and *Drosophila* sequences have longer aminoterminal regions but they do not show significant homology to the aminoterminal region of PRKAG3 and were not included.

Abbreviations:

*: stop codon

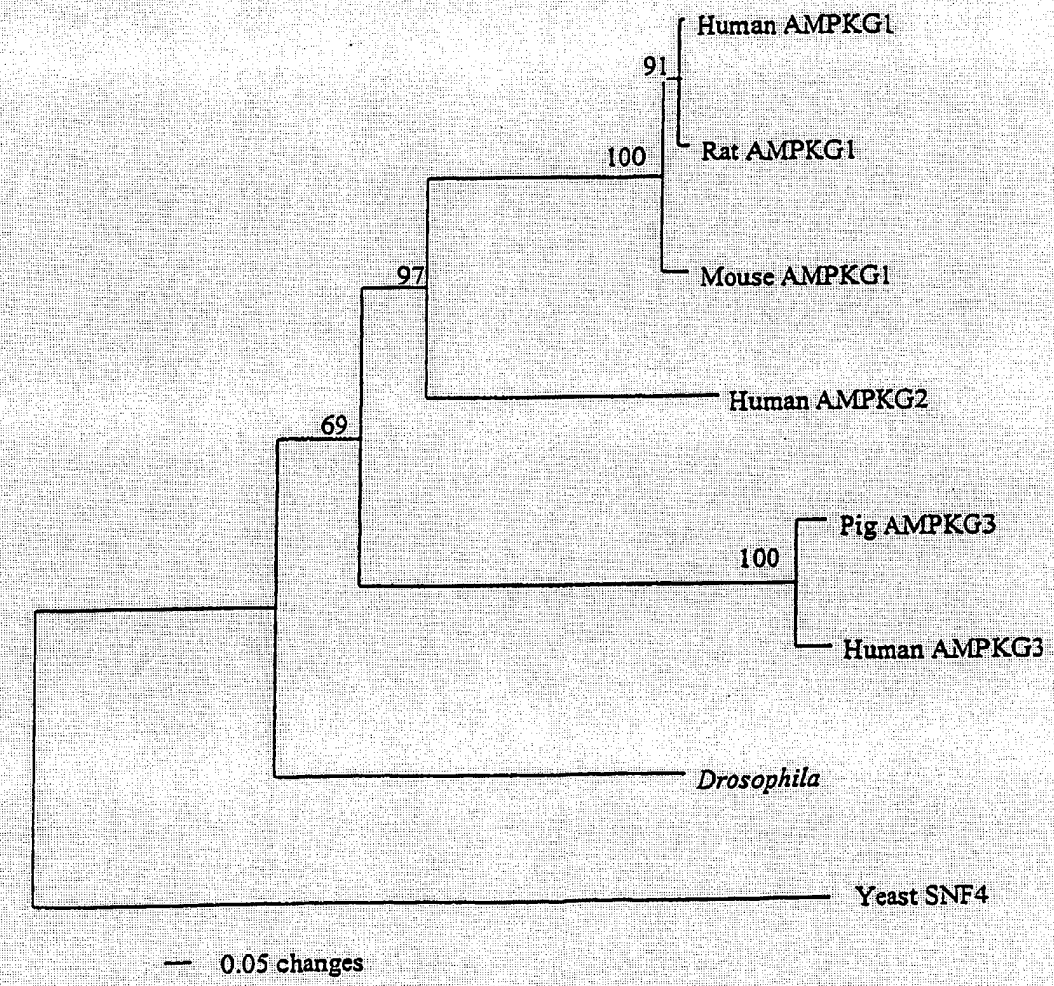
'-': identity to master sequence

'.': alignment gap

The four CBS domains are overlined and the position of the *RN*⁻ mutation is indicated by an arrow.

Table 2 below shows the amino acid (above diagonal) and nucleotide sequence (below diagonal) identities (in %) among mammalian, *Drosophila* and yeast AMPKG/SNF4 sequences. In the case of pig PRKAG3 and human PRKAG3, the identities were calculated referring to the portions thereof represented respectively by SEQ ID NO: 1 and SEQ ID NO: 3, for the nucleotide sequences, and by SEQ ID NO: 2 and SEQ ID NO: 4, for the amino acid sequences.

Figure 3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Rat AMPKG1
100
97
Mouse AMPKG1
Human AMPKG2
69
Pig AMPKG3
100
Human AMPKG3
Drosophila
Yeast SNF4
0.05 changes

Figure 4

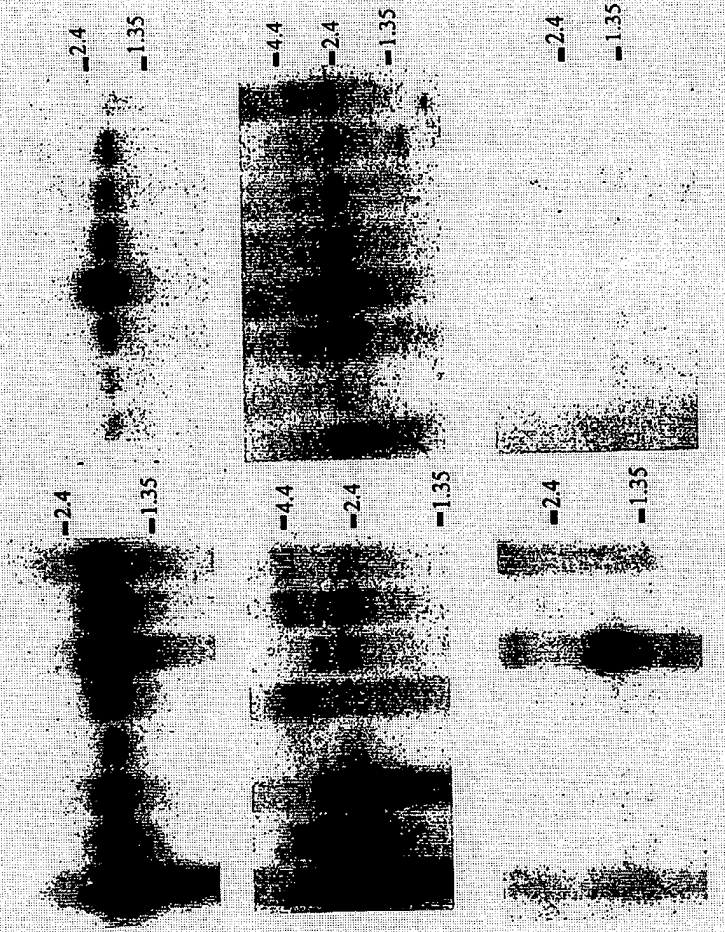

Figure 5

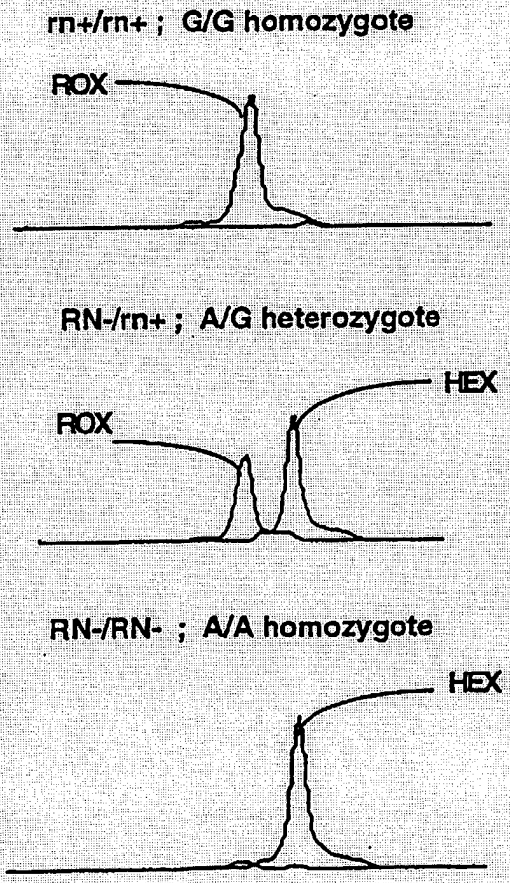
rn+/rn+ ; G/G homozygote
ROX
RN-/rn+ ; A/G heterozygote
HEX
ROX
RN-/RN- ; A/A homozygote
HEX

Figure 6

SEQUENCE LISTING

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MILAN, Denis
ANDERSSON, Leif
LOOFT, Christian
ROBIC, Annie
ROGEL-GAILLARD, Claire
IANNUCCELLI, Nathalie
GELLIN, Joël
KALM, Ernst
LE ROY, Pascale
CHARDON, Patrick

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gaaggggggc ctccgggccc gagggaaggt ccccagtcca ggccagttgc tgagtccacc 180

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gatgagcttg ggctggtgga agagaagcca gccccgtgcc catccccaga ggtgctgtta 420

cccaggctgg gctgggatga tgagctgcag aagccggggg cccaggtcta c atg cac 477
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His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys Phe Lys
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 35                  40                  45                  50

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Gln Ser Phe Val Gly Met Leu Thr Ile Thr Asp Phe Ile Leu Val Leu
                 55                  60                  65

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His Arg Tyr Tyr Arg Ser Pro Leu Val Gln Ile Tyr Glu Ile Glu Gln
             70                  75                  80

cat aag att gag acc tgg agg gag atc tac ctg caa ggc tgc ttc aag    765
His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys Phe Lys
         85                  90                  95

cct ctg gtc tcc atc tct cct aat gat agc ctg ttt gaa gct gtc tac    813
Pro Leu Val Ser Ile Ser Pro Asn Asp Ser Leu Phe Glu Ala Val Tyr
    100                 105                 110

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115                 120                 125                 130

tca ggc aac gta ctc cac atc ctc aca cac aaa cgc ctg ctc aag ttc    909
Ser Gly Asn Val Leu His Ile Leu Thr His Lys Arg Leu Leu Lys Phe
                135                 140                 145

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Leu His Ile Phe Gly Ser Leu Leu Pro Arg Pro Ser Phe Leu Tyr Arg
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ctg gag aca gca ccc atc ctg act gca ctg gac atc ttt gtg gac cgg   1053
Leu Glu Thr Ala Pro Ile Leu Thr Ala Leu Asp Ile Phe Val Asp Arg
    180                 185                 190

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Arg Val Ser Ala Leu Pro Val Val Asn Glu Cys Gly Gln Val Val Gly
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Cys Leu Glu Gly Val Leu Ser Cys Gln Pro His Glu Ser Leu Gly Glu
        245                 250                 255

gtg atc gac agg att gct cgg gag cag gta cac agg ctg gtg cta gtg   1293
Val Ile Asp Arg Ile Ala Arg Glu Gln Val His Arg Leu Val Leu Val
    260                 265                 270

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  1               5                  10                  15

Ser Lys Leu Val Ile Phe Asp Thr Met Leu Glu Ile Lys Lys Ala Phe
            20                  25                  30

Phe Ala Leu Val Ala Asn Gly Val Arg Ala Ala Pro Leu Trp Asp Ser
        35                  40                  45
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Lys Lys Gln Ser Phe Val Gly Met Leu Thr Ile Thr Asp Phe Ile Leu
 50                  55                  60

Val Leu His Arg Tyr Tyr Arg Ser Pro Leu Val Gln Ile Tyr Glu Ile
 65                  70                  75                  80

Glu Gln His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys
                 85                  90                  95

Phe Lys Pro Leu Val Ser Ile Ser Pro Asn Asp Ser Leu Phe Glu Ala
            100                 105                 110

Val Tyr Thr Leu Ile Lys Asn Arg Ile His Arg Leu Pro Val Leu Asp
        115                 120                 125

Pro Val Ser Gly Asn Val Leu His Ile Leu Thr His Lys Arg Leu Leu
    130                 135                 140

Lys Phe Leu His Ile Phe Gly Ser Leu Leu Pro Arg Pro Ser Phe Leu
145                 150                 155                 160

Tyr Arg Thr Ile Gln Asp Leu Gly Ile Gly Thr Phe Arg Asp Leu Ala
                165                 170                 175

Val Val Leu Glu Thr Ala Pro Ile Leu Thr Ala Leu Asp Ile Phe Val
            180                 185                 190

Asp Arg Arg Val Ser Ala Leu Pro Val Val Asn Glu Cys Gly Gln Val
        195                 200                 205

Val Gly Leu Tyr Ser Arg Phe Asp Val Ile His Leu Ala Ala Gln Gln
    210                 215                 220

Thr Tyr Asn His Leu Asp Met Ser Val Gly Glu Ala Leu Arg Gln Arg
225                 230                 235                 240

Thr Leu Cys Leu Glu Gly Val Leu Ser Cys Gln Pro His Glu Ser Leu
                245                 250                 255

Gly Glu Val Ile Asp Arg Ile Ala Arg Glu Gln Val His Arg Leu Val
            260                 265                 270

Leu Val Asp Glu Thr Gln His Leu Leu Gly Val Val Ser Leu Ser Asp
        275                 280                 285

Ile Leu Gln Ala Leu Val Leu Ser Pro Ala Gly Ile Asp Ala Leu Gly
    290                 295                 300

Ala
305
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<212> DNA
<213> Sus scrofa

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ggaatttcaa gtcagccaac 20

<210> 6
<211> 20
<212> DNA
<213> Sus scrofa

<400> 6
cttcaaaaga ccgtgctact 20

<210> 7
<211> 20
<212> DNA
<213> Sus scrofa

<400> 7
ctgggaacct ctatatgctg 20

<210> 8
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<213> Sus scrofa

<400> 8
tagggaaata caaatcacag 20

<210> 9
<211> 20
<212> DNA
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<400> 9
ctccagctca caggatgaca 20

<210> 10
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<212> DNA
<213> Sus scrofa

<400> 10
gtttctgcag ctttagcatc tattcc 26

<210> 11
<211> 20
<212> DNA
<213> Sus scrofa

<400> 11
gaagtatcct gggcttctga 20

<210> 12
<211> 26
<212> DNA
<213> Sus scrofa

<400> 12

gtttctccag gtttccagac atccac 26

<210> 13
<211> 20
<212> DNA
<213> Sus scrofa

<400> 13
gcttctgtct gcccctactt 20

<210> 14
<211> 26
<212> DNA
<213> Sus scrofa

<400> 14
gtttctaagt tctactgtaa gacacc 26

<210> 15
<211> 20
<212> DNA
<213> Sus scrofa

<400> 15
ccaagctgtg gtggctgaat 20

<210> 16
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<400> 16
cagcacagca gtgccaccta 20

<210> 17
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<400> 17
caaactcttc taggcgtgt 19

<210> 18
<211> 26
<212> DNA
<213> Sus scrofa

<400> 18
gtttctggaa cttccatatg ccatgg 26

<210> 19
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<212> DNA
<213> Sus scrofa

<400> 19
agggtggatg gtaggcttca 20

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<400> 20
gtctcgctcc tgaaggaagt 20

<210> 21
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<400> 21
agtcacgtgg ccatgctatc 20

<210> 22
<211> 20
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<400> 22
ctcaactgga ttgagtcagt 20

<210> 23
<211> 20
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<213> Sus scrofa

<400> 23
ttggcgcaac tgttatttct 20

<210> 24
<211> 19
<212> DNA
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<400> 24
aggcaaagga agagcacag 19

<210> 25
<211> 18
<212> DNA
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<400> 25
agccgtgggc atcgttgg 18

<210> 26
<211> 21

<212> DNA
<213> Sus scrofa

<400> 26
agaaggagac agacagggcga 21

<210> 27
<211> 1873
<212> ADN
<213> Sus scrofa

<220>
<221> CDS
<222> (1)..(1395)

<400> 27

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atg agc ttc cta gag caa gga gag agc cgt tca tgg cca tcc cga gct   48
Met Ser Phe Leu Glu Gln Gly Glu Ser Arg Ser Trp Pro Ser Arg Ala
 1               5                  10                  15

gta acc acc agc tca gaa aga agc cat ggg gac cag ggg aac aag gcc   96
Val Thr Thr Ser Ser Glu Arg Ser His Gly Asp Gln Gly Asn Lys Ala
            20                  25                  30

tct aga tgg aca agg cag gag gat gta gag gaa ggg ggg cct ccg ggc  144
Ser Arg Trp Thr Arg Gln Glu Asp Val Glu Glu Gly Gly Pro Pro Gly
        35                  40                  45

ccg agg gaa ggt ccc cag tcc agg cca gtt gct gag tcc acc ggg cag  192
Pro Arg Glu Gly Pro Gln Ser Arg Pro Val Ala Glu Ser Thr Gly Gln
    50                  55                  60

gag gcc aca ttc ccc aag gcc aca ccc ttg gcc caa gcc gct ccc ttg  240
Glu Ala Thr Phe Pro Lys Ala Thr Pro Leu Ala Gln Ala Ala Pro Leu
65                  70                  75                  80

gcc gag gtg gac aac ccc cca aca gag cgg gac atc ctc ccc tct gac  288
Ala Glu Val Asp Asn Pro Pro Thr Glu Arg Asp Ile Leu Pro Ser Asp
                85                  90                  95

tgt gca gcc tca gcc tcc gac tcc aac aca gac cat ctg gat ctg ggc  336
Cys Ala Ala Ser Ala Ser Asp Ser Asn Thr Asp His Leu Asp Leu Gly
            100                 105                 110

ata gag ttc tca gcc tcg gcg gcg tcg ggg gat gag ctt ggg ctg gtg  384
Ile Glu Phe Ser Ala Ser Ala Ala Ser Gly Asp Glu Leu Gly Leu Val
        115                 120                 125

gaa gag aag cca gcc ccg tgc cca tcc cca gag gtg ctg tta ccc agg  432
Glu Glu Lys Pro Ala Pro Cys Pro Ser Pro Glu Val Leu Leu Pro Arg
    130                 135                 140

ctg ggc tgg gat gat gag ctg cag aag ccg ggg gcc cag gtc tac atg  480
Leu Gly Trp Asp Asp Glu Leu Gln Lys Pro Gly Ala Gln Val Tyr Met
145                 150                 155                 160

cac ttc atg cag gag cac acc tgc tac gat gcc atg gcg acc agc tcc  528
His Phe Met Gln Glu His Thr Cys Tyr Asp Ala Met Ala Thr Ser Ser
                165                 170                 175
```

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aaa ctg gtc atc ttc gac acc atg ctg gag atc aag aag gcc ttc ttt   576
Lys Leu Val Ile Phe Asp Thr Met Leu Glu Ile Lys Lys Ala Phe Phe
            180                 185                 190

gcc ctg gtg gcc aac ggc gtc cga gcg gca cct ttg tgg gac agc aag   624
Ala Leu Val Ala Asn Gly Val Arg Ala Ala Pro Leu Trp Asp Ser Lys
        195                 200                 205

aag cag agc ttc gtg ggg atg ctg acc atc aca gac ttc atc ttg gtg   672
Lys Gln Ser Phe Val Gly Met Leu Thr Ile Thr Asp Phe Ile Leu Val
    210                 215                 220

ctg cac cgc tat tac agg tcc ccc ctg gtc cag atc tac gag att gaa   720
Leu His Arg Tyr Tyr Arg Ser Pro Leu Val Gln Ile Tyr Glu Ile Glu
225                 230                 235                 240

gaa cat aag att gag acc tgg agg gag atc tac ctt caa ggc tgc ttc   768
Glu His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys Phe
                245                 250                 255

aag cct ctg gtc tcc atc tct ccc aat gac agc ctg ttc gaa gct gtc   816
Lys Pro Leu Val Ser Ile Ser Pro Asn Asp Ser Leu Phe Glu Ala Val
            260                 265                 270

tac gcc ctc atc aag aac cgg atc cac cgc ctg ccg gtc ctg gac cct   864
Tyr Ala Leu Ile Lys Asn Arg Ile His Arg Leu Pro Val Leu Asp Pro
        275                 280                 285

gtc tcc ggg gct gtg ctc cac atc ctc aca cat aag cgg ctt ctc aag   912
Val Ser Gly Ala Val Leu His Ile Leu Thr His Lys Arg Leu Leu Lys
    290                 295                 300

ttc ctg cac atc ttt ggc acc ctg ctg ccc cgg ccc tcc ttc ctc tac   960
Phe Leu His Ile Phe Gly Thr Leu Leu Pro Arg Pro Ser Phe Leu Tyr
305                 310                 315                 320

cgc acc atc caa gat ttg ggc atc ggc aca ttc cga gac ttg gcc gtg  1008
Arg Thr Ile Gln Asp Leu Gly Ile Gly Thr Phe Arg Asp Leu Ala Val
                325                 330                 335

gtg ctg gaa acg gcg ccc atc ctg acc gca ctg gac atc ttc gtg gac  1056
Val Leu Glu Thr Ala Pro Ile Leu Thr Ala Leu Asp Ile Phe Val Asp
            340                 345                 350

cgg cgt gtg tct gcg ctg cct gtg gtc aac gaa act gga cag gta gtg  1104
Arg Arg Val Ser Ala Leu Pro Val Val Asn Glu Thr Gly Gln Val Val
        355                 360                 365

ggc ctc tac tct cgc ttt gat gtg atc cac ctg gct gcc caa caa aca  1152
Gly Leu Tyr Ser Arg Phe Asp Val Ile His Leu Ala Ala Gln Gln Thr
    370                 375                 380

tac aac cac ctg gac atg aat gtg gga gaa gcc ctg agg cag cgg aca  1200
Tyr Asn His Leu Asp Met Asn Val Gly Glu Ala Leu Arg Gln Arg Thr
385                 390                 395                 400

ctg tgt ctg gaa ggc gtc ctt tcc tgc cag ccc cac gag acc ttg ggg  1248
Leu Cys Leu Glu Gly Val Leu Ser Cys Gln Pro His Glu Thr Leu Gly
                405                 410                 415
```

```
gaa gtc att gac cgg att gtc cgg gaa cag gtg cac cgc ctg gtg ctc   1296
Glu Val Ile Asp Arg Ile Val Arg Glu Gln Val His Arg Leu Val Leu
            420                 425                 430

gtg gat gag acc cag cac ctt ctg ggc gtg gtg tcc ctc tct gac atc   1344
Val Asp Glu Thr Gln His Leu Leu Gly Val Val Ser Leu Ser Asp Ile
        435                 440                 445

ctt cag gct ctg gtg ctc agc cct gct gga att gat gcc ctc ggg gcc   1392
Leu Gln Ala Leu Val Leu Ser Pro Ala Gly Ile Asp Ala Leu Gly Ala
    450                 455                 460

tga gaaccttgga acctttgctc tcaggccacc tggcacacct ggaagccagt        1445

465

gaagggagcc gtggactcag ctctcacttc ccctcagccc cacttgctgg tctggctctt 1505

gttcaggtag gctccgcccg gggcccctgg cctcagcatc agcccctcag tctccctggg 1565

cacccagatc tcagactggg gcaccctgaa gatgggagtg gcccagctta tagctgagca 1625

gccttgtgaa atctaccagc atcaagactc actgtgggac cactgctttg tcccattctc 1685

agctgaaatg atggagggcc tcataagagg ggtggacagg gcctggagta gaggccagat 1745

cagtgacgtg ccttcaggac ctccggggag ttagagctgc cctctctcag ttcagttccc 1805

ccctgctgag aatgtccctg gaaggaagcc agttaataaa ccttggttgg atggaatttg 1865

gagagtcg                                                          1873


<210> 28
<211> 464
<212> PRT
<213> Sus scrofa

<400> 28
Met Ser Phe Leu Glu Gln Gly Glu Ser Arg Ser Trp Pro Ser Arg Ala
  1               5                  10                  15
Val Thr Thr Ser Ser Glu Arg Ser His Gly Asp Gln Gly Asn Lys Ala
            20                  25                  30
Ser Arg Trp Thr Arg Gln Glu Asp Val Glu Glu Gly Gly Pro Pro Gly
        35                  40                  45
Pro Arg Glu Gly Pro Gln Ser Arg Pro Val Ala Glu Ser Thr Gly Gln
    50                  55                  60
Glu Ala Thr Phe Pro Lys Ala Thr Pro Leu Ala Gln Ala Ala Pro Leu
65                  70                  75                  80
Ala Glu Val Asp Asn Pro Pro Thr Glu Arg Asp Ile Leu Pro Ser Asp
                85                  90                  95
Cys Ala Ala Ser Ala Ser Asp Ser Asn Thr Asp His Leu Asp Leu Gly
            100                 105                 110
Ile Glu Phe Ser Ala Ser Ala Ala Ser Gly Asp Glu Leu Gly Leu Val
        115                 120                 125
Glu Glu Lys Pro Ala Pro Cys Pro Ser Pro Glu Val Leu Leu Pro Arg
    130                 135                 140
Leu Gly Trp Asp Asp Glu Leu Gln Lys Pro Gly Ala Gln Val Tyr Met
145                 150                 155                 160
His Phe Met Gln Glu His Thr Cys Tyr Asp Ala Met Ala Thr Ser Ser
                165                 170                 175
```

```
Lys Leu Val Ile Phe Asp Thr Met Leu Glu Ile Lys Lys Ala Phe Phe
            180                 185                 190
Ala Leu Val Ala Asn Gly Val Arg Ala Ala Pro Leu Trp Asp Ser Lys
        195                 200                 205
Lys Gln Ser Phe Val Gly Met Leu Thr Ile Thr Asp Phe Ile Leu Val
    210                 215                 220
Leu His Arg Tyr Tyr Arg Ser Pro Leu Val Gln Ile Tyr Glu Ile Glu
225                 230                 235                 240
Glu His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys Phe
                245                 250                 255
Lys Pro Leu Val Ser Ile Ser Pro Asn Asp Ser Leu Phe Glu Ala Val
            260                 265                 270
Tyr Ala Leu Ile Lys Asn Arg Ile His Arg Leu Pro Val Leu Asp Pro
        275                 280                 285
Val Ser Gly Ala Val Leu His Ile Leu Thr His Lys Arg Leu Leu Lys
    290                 295                 300
Phe Leu His Ile Phe Gly Thr Leu Leu Pro Arg Pro Ser Phe Leu Tyr
305                 310                 315                 320
Arg Thr Ile Gln Asp Leu Gly Ile Gly Thr Phe Arg Asp Leu Ala Val
                325                 330                 335
Val Leu Glu Thr Ala Pro Ile Leu Thr Ala Leu Asp Ile Phe Val Asp
            340                 345                 350
Arg Arg Val Ser Ala Leu Pro Val Val Asn Glu Thr Gly Gln Val Val
        355                 360                 365
Gly Leu Tyr Ser Arg Phe Asp Val Ile His Leu Ala Ala Gln Gln Thr
    370                 375                 380
Tyr Asn His Leu Asp Met Asn Val Gly Glu Ala Leu Arg Gln Arg Thr
385                 390                 395                 400
Leu Cys Leu Glu Gly Val Leu Ser Cys Gln Pro His Glu Thr Leu Gly
                405                 410                 415
Glu Val Ile Asp Arg Ile Val Arg Glu Gln Val His Arg Leu Val Leu
            420                 425                 430
Val Asp Glu Thr Gln His Leu Leu Gly Val Val Ser Leu Ser Asp Ile
        435                 440                 445
Leu Gln Ala Leu Val Leu Ser Pro Ala Gly Ile Asp Ala Leu Gly Ala
    450                 455                 460
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<210> 29
<211> 2115
<212> ADN
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(1395)

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<400> 29
atg agc ttc cta gag caa gaa aac agc agc tca tgg cca tca cca gct   48
Met Ser Phe Leu Glu Gln Glu Asn Ser Ser Ser Trp Pro Ser Pro Ala
  1               5                  10                  15

gtg acc agc agc tca gaa aga atc cgt ggg aaa cgg agg gcc aaa gcc   96
Val Thr Ser Ser Ser Glu Arg Ile Arg Gly Lys Arg Arg Ala Lys Ala
            20                  25                  30

ttg aga tgg aca agg cag aag tcg gtg gag gaa ggg gag cca cca ggt   144
Leu Arg Trp Thr Arg Gln Lys Ser Val Glu Glu Gly Glu Pro Pro Gly
        35                  40                  45
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cag ggg gaa ggt ccc cgg tcc agg cca act gct gag tcc acc ggg ctg   192
Gln Gly Glu Gly Pro Arg Ser Arg Pro Thr Ala Glu Ser Thr Gly Leu
     50                  55                  60

gag gcc aca ttc ccc aag acc aca ccc ttg gct caa gct gat cct gcc   240
Glu Ala Thr Phe Pro Lys Thr Thr Pro Leu Ala Gln Ala Asp Pro Ala
 65                  70                  75                  80

ggg gtg ggc act cca cca aca ggg tgg gac tgc ctc ccc tct gac tgt   288
Gly Val Gly Thr Pro Pro Thr Gly Trp Asp Cys Leu Pro Ser Asp Cys
                 85                  90                  95

aca gcc tca gct gca ggc tcc agc aca gat gat gtg gag ctg gcc acg   336
Thr Ala Ser Ala Ala Gly Ser Ser Thr Asp Asp Val Glu Leu Ala Thr
            100                 105                 110

gag ttc cca gcc aca gag gcc tgg gag tgt gag cta gaa ggc ctg ctg   384
Glu Phe Pro Ala Thr Glu Ala Trp Glu Cys Glu Leu Glu Gly Leu Leu
        115                 120                 125

gaa gag agg cct gcc ctg tgc ctg tcc ccg cag gcc cca ttt ccc aag   432
Glu Glu Arg Pro Ala Leu Cys Leu Ser Pro Gln Ala Pro Phe Pro Lys
    130                 135                 140

ctg ggc tgg gat gac gaa ctg cgg aaa ccc ggc gcc cag atc tac atg   480
Leu Gly Trp Asp Asp Glu Leu Arg Lys Pro Gly Ala Gln Ile Tyr Met
145                 150                 155                 160

cgc ttc atg cag gag cac acc tgc tac gat gcc atg gca act agc tcc   528
Arg Phe Met Gln Glu His Thr Cys Tyr Asp Ala Met Ala Thr Ser Ser
                165                 170                 175

aag cta gtc atc ttc gac acc atg ctg gag atc aag aag gcc ttc ttt   576
Lys Leu Val Ile Phe Asp Thr Met Leu Glu Ile Lys Lys Ala Phe Phe
            180                 185                 190

gct ctg gtg gcc aac ggt gtg cgg gca gcc cct cta tgg gac agc aag   624
Ala Leu Val Ala Asn Gly Val Arg Ala Ala Pro Leu Trp Asp Ser Lys
        195                 200                 205

aag cag agc ttt gtg ggg atg ctg acc atc act gac ttc atc ctg gtg   672
Lys Gln Ser Phe Val Gly Met Leu Thr Ile Thr Asp Phe Ile Leu Val
    210                 215                 220

ctg cat cgc tac tac agg tcc ccc ctg gtc cag atc tat gag att gaa   720
Leu His Arg Tyr Tyr Arg Ser Pro Leu Val Gln Ile Tyr Glu Ile Glu
225                 230                 235                 240

caa cat aag att gag acc tgg agg gag atc tac ctg caa ggc tgc ttc   768
Gln His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys Phe
                245                 250                 255

aag cct ctg gtc tcc atc tct cct aat gat agc ctg ttt gaa gct gtc   816
Lys Pro Leu Val Ser Ile Ser Pro Asn Asp Ser Leu Phe Glu Ala Val
            260                 265                 270

tac acc ctc atc aag aac cgg atc cat cgc ctg cct gtt ctt gac ccg   864
Tyr Thr Leu Ile Lys Asn Arg Ile His Arg Leu Pro Val Leu Asp Pro
        275                 280                 285
```

```
gtg tca ggc aac gta ctc cac atc ctc aca cac aaa cgc ctg ctc aag   912
Val Ser Gly Asn Val Leu His Ile Leu Thr His Lys Arg Leu Leu Lys
    290                 295                 300

ttc ctg cac atc ttt ggt tcc ctg ctg ccc cgg ccc tcc ttc ctc tac   960
Phe Leu His Ile Phe Gly Ser Leu Leu Pro Arg Pro Ser Phe Leu Tyr
305                 310                 315                 320

cgc act atc caa gat ttg ggc atc ggc aca ttc cga gac ttg gct gtg  1008
Arg Thr Ile Gln Asp Leu Gly Ile Gly Thr Phe Arg Asp Leu Ala Val
                325                 330                 335

gtg ctg gag aca gca ccc atc ctg act gca ctg gac atc ttt gtg gac  1056
Val Leu Glu Thr Ala Pro Ile Leu Thr Ala Leu Asp Ile Phe Val Asp
            340                 345                 350

cgg cgt gtg tct gca ctg cct gtg gtc aac gaa tgt ggt cag gtc gtg  1104
Arg Arg Val Ser Ala Leu Pro Val Val Asn Glu Cys Gly Gln Val Val
        355                 360                 365

ggc ctc tat tcc cgc ttt gat gtg att cac ctg gct gcc cag caa acc  1152
Gly Leu Tyr Ser Arg Phe Asp Val Ile His Leu Ala Ala Gln Gln Thr
    370                 375                 380

tac aac cac ctg gac atg agt gtg gga gaa gcc ctg agg cag agg aca  1200
Tyr Asn His Leu Asp Met Ser Val Gly Glu Ala Leu Arg Gln Arg Thr
385                 390                 395                 400

cta tgt ctg gag gga gtc ctt tcc tgc cag ccc cac gag agc ttg ggg  1248
Leu Cys Leu Glu Gly Val Leu Ser Cys Gln Pro His Glu Ser Leu Gly
                405                 410                 415

gaa gtg atc gac agg att gct cgg gag cag gta cac agg ctg gtg cta  1296
Glu Val Ile Asp Arg Ile Ala Arg Glu Gln Val His Arg Leu Val Leu
            420                 425                 430

gtg gac gag acc cag cat ctc ttg ggc gtg gtc tcc ctc tcc gac atc  1344
Val Asp Glu Thr Gln His Leu Leu Gly Val Val Ser Leu Ser Asp Ile
        435                 440                 445

ctt cag gca ctg gtg ctc agc cct gct ggc atc gat gcc ctc ggg gcc  1392
Leu Gln Ala Leu Val Leu Ser Pro Ala Gly Ile Asp Ala Leu Gly Ala
    450                 455                 460

tga gaagatctga gtcctcaatc ccaagccaac tgcacactgg aagccaatga       1445

465

aggaattgag aacagcttca tttccccaac cccaatttgc tggttcagct atgattcagg 1505

cttcttcagc cttccaaaat tgcctttgcc ttacttgtgc tcccagaacc cttcgggcat 1565

gcccagtgca ccatgggatg atgaaattaa ggagaacagc tgagtcaagc ttggaggtcc 1625

ctgaaccaga ggcactagga ttaccccagg gccatctgtg ctccatgccc gcccatcccc 1685

ttgccgcctg actgggtcgg atggccccag tgggtttagt cagggcttct ggattcctcg 1745

gtttctgggc tacctatggc ttcagccttc agctcctggg agtcccagct gttgttccca 1805

gcaacgtcgc cactgccctc ctactctcca ggctttgtca tttcaaggct gctgaaatgc 1865
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tgcatttcag gggccaccat ggagcagccg ttatttatag aactgcctgt tggaggtggg 1925

gagtcctccc tccattcttg tccagaaaac tccttagctc tcgcagtgag ccatgttctt 1985

agtctccagg gatggatggc cttgtatatg gacccctgag aatgagcaat tgagaaaaca 2045

aaacaaaagg aacaatccat gaacttagat tttattggtt tcactcaaaa tgctgcagtc 2105

atttgacctg 2115

<210> 30
<211> 464
<212> PRT
<213> Homo sapiens

<400> 30

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Met Ser Phe Leu Glu Gln Glu Asn Ser Ser Ser Trp Pro Ser Pro Ala
 1               5                  10                  15
Val Thr Ser Ser Ser Glu Arg Ile Arg Gly Lys Arg Arg Ala Lys Ala
            20                  25                  30
Leu Arg Trp Thr Arg Gln Lys Ser Val Glu Glu Gly Glu Pro Pro Gly
        35                  40                  45
Gln Gly Glu Gly Pro Arg Ser Arg Pro Thr Ala Glu Ser Thr Gly Leu
    50                  55                  60
Glu Ala Thr Phe Pro Lys Thr Thr Pro Leu Ala Gln Ala Asp Pro Ala
65                  70                  75                  80
Gly Val Gly Thr Pro Pro Thr Gly Trp Asp Cys Leu Pro Ser Asp Cys
                85                  90                  95
Thr Ala Ser Ala Ala Gly Ser Ser Thr Asp Asp Val Glu Leu Ala Thr
            100                 105                 110
Glu Phe Pro Ala Thr Glu Ala Trp Glu Cys Glu Leu Glu Gly Leu Leu
        115                 120                 125
Glu Glu Arg Pro Ala Leu Cys Leu Ser Pro Gln Ala Pro Phe Pro Lys
    130                 135                 140
Leu Gly Trp Asp Asp Glu Leu Arg Lys Pro Gly Ala Gln Ile Tyr Met
145                 150                 155                 160
Arg Phe Met Gln Glu His Thr Cys Tyr Asp Ala Met Ala Thr Ser Ser
                165                 170                 175
Lys Leu Val Ile Phe Asp Thr Met Leu Glu Ile Lys Lys Ala Phe Phe
            180                 185                 190
Ala Leu Val Ala Asn Gly Val Arg Ala Ala Pro Leu Trp Asp Ser Lys
        195                 200                 205
Lys Gln Ser Phe Val Gly Met Leu Thr Ile Thr Asp Phe Ile Leu Val
    210                 215                 220
Leu His Arg Tyr Tyr Arg Ser Pro Leu Val Gln Ile Tyr Glu Ile Glu
225                 230                 235                 240
Gln His Lys Ile Glu Thr Trp Arg Glu Ile Tyr Leu Gln Gly Cys Phe
                245                 250                 255
Lys Pro Leu Val Ser Ile Ser Pro Asn Asp Ser Leu Phe Glu Ala Val
            260                 265                 270
Tyr Thr Leu Ile Lys Asn Arg Ile His Arg Leu Pro Val Leu Asp Pro
        275                 280                 285
Val Ser Gly Asn Val Leu His Ile Leu Thr His Lys Arg Leu Leu Lys
    290                 295                 300
Phe Leu His Ile Phe Gly Ser Leu Leu Pro Arg Pro Ser Phe Leu Tyr
305                 310                 315                 320
Arg Thr Ile Gln Asp Leu Gly Ile Gly Thr Phe Arg Asp Leu Ala Val
                325                 330                 335
```

```
Val Leu Glu Thr Ala Pro Ile Leu Thr Ala Leu Asp Ile Phe Val Asp
            340                 345                 350
Arg Arg Val Ser Ala Leu Pro Val Val Asn Glu Cys Gly Gln Val Val
        355                 360                 365
Gly Leu Tyr Ser Arg Phe Asp Val Ile His Leu Ala Ala Gln Gln Thr
    370                 375                 380
Tyr Asn His Leu Asp Met Ser Val Gly Glu Ala Leu Arg Gln Arg Thr
385                 390                 395                 400
Leu Cys Leu Glu Gly Val Leu Ser Cys Gln Pro His Glu Ser Leu Gly
                405                 410                 415
Glu Val Ile Asp Arg Ile Ala Arg Glu Gln Val His Arg Leu Val Leu
            420                 425                 430
Val Asp Glu Thr Gln His Leu Leu Gly Val Val Ser Leu Ser Asp Ile
        435                 440                 445
Leu Gln Ala Leu Val Leu Ser Pro Ala Gly Ile Asp Ala Leu Gly Ala
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35 40 45

Gln Glu Met Ser Phe Leu Glu Gln Gly Glu Ser Arg Ser Trp Pro Ser
50 55 60

Arg Ala Val Thr Thr Ser Ser Glu Arg Ser His Gly Asp Gln Gly Asn
65 70 75 80

Lys Ala Ser Arg Trp Thr Arg Gln Glu Asp Val Glu Glu Gly Gly Pro
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Gly Gln Glu Ala Thr Phe Pro Lys Ala Thr Pro Leu Ala Gln Ala Ala
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Pro Leu Ala Glu Val Asp Asn Pro Pro Thr Glu Arg Asp Ile Leu Pro
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Ser Asp Cys Ala Ala Ser Ala Ser Asp Ser Asn Thr Asp His Leu Asp
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Leu Gly Ile Glu Phe Ser Ala Ser Ala Ala Ser Gly Asp Glu Leu Gly
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Leu Val Glu Glu Lys Pro Ala Pro Cys Pro Ser Pro Glu Val Leu Leu
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Pro Arg Leu Gly Trp Asp Asp Glu Leu Gln Lys Pro Gly Ala Gln Val
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Tyr Met His Phe Met Gln Glu His Thr Cys Tyr Asp Ala Met Ala Thr
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Phe Phe Ala Leu Val Ala Asn Gly Val Arg Ala Ala Pro Leu Trp Asp
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Ser Lys Lys Gln Ser Phe Val Gly Met Leu Thr Ile Thr Asp Phe Ile
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Ala Val Tyr Ala Leu Ile Lys Asn Arg Ile His Arg Leu Pro Val Leu
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Asp Pro Val Ser Gly Ala Val Leu His Ile Leu Thr His Lys Arg Leu
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Leu Lys Phe Leu His Ile Phe Gly Thr Leu Leu Pro Arg Pro Ser Phe
        355                 360                 365

Leu Tyr Arg Thr Ile Gln Asp Leu Gly Ile Gly Thr Phe Arg Asp Leu
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Ala Val Val Leu Glu Thr Ala Pro Ile Leu Thr Ala Leu Asp Ile Phe
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Val Val Gly Leu Tyr Ser Arg Phe Asp Val Ile His Leu Ala Ala Gln
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Gln Thr Tyr Asn His Leu Asp Met Asn Val Gly Glu Ala Leu Arg Gln
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Arg Thr Leu Cys Leu Glu Gly Val Leu Ser Cys Gln Pro His Glu Thr
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PC./EP 00/09896

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N15/11 C12N9/12 C12Q1/68 A01K67/027
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
STRAND, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER ET AL.: "WashU-NCI human EST project" EMBL DATABASE ACC NO: AA178898, 1 January 1997 (1997-01-01), XP002130593 cited in the application abstract	1-5, 11-17
X	ROBIC ET AL.: "A radiation hybrid map of the RN region in pigs demonstrates conserved gene order compared with the human and mouse genomes" MAMMALIAN GENOME, vol. 10, no. 6, June 1999 (1999-06), pages 565-568, XP000876695 cited in the application page 565	29-33
A	page 567; figure 1; table 1	1-28, 32-37

-/--

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 March 2001	19/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van Klompenburg, W

1

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INTERNATIONAL SEARCH REPORT

International Application No
PC../EP 00/09896

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 25341 A (ST VINCENTS INST MED RES ;DARTMOUTH COLLEGE (US); KEMP BRUCE E (AU) 17 July 1997 (1997-07-17) page 18, line 21 - line 34 page 25, line 1 -page 28, line 5	13-15
A	claims 16-21	1-12, 16-37

X	WO 98 58052 A (INCYTE PHARMA INC ;CORLEY NEIL C (US); BANDMAN OLGA (US); GOLI SUR) 23 December 1998 (1998-12-23) SEQ ID NOs 7 & 14 page 18, line 21 -page 19, line 8 page 38, line 6 -page 44, line 29	13-15
A	claims 1-24; figure 7	1-12, 16-37

X	WATERSTON: "Homo sapiens chromosome unknown clone NH0459I19" EMBL DATABASE ACC NO: AC009974, 9 September 1999 (1999-09-09), XP002130594 abstract	11-16

X	HILLIER ET AL.: "The WashU-Merck EST Project" EMBL DATABASE ACC NO: W94830, 17 July 1996 (1996-07-17), XP002130595 abstract	11-16

A	MILAN ET AL.: "Accurate mapping of the "acid meat" RN gene on genetic and physical maps of pig chromosome 15" MAMMALIAN GENOME, vol. 7, no. 1, January 1996 (1996-01), pages 47-51, XP000876743 cited in the application page 50, column 2 -page 51, column 1; figure 1	1-35

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INTERNATIONAL SEARCH REPORT

International Application No
PC./EP 00/09896

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CHEUNG ET AL.: "Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding" BIOCHEMICAL JOURNAL, vol. 346, no. 3, 15 March 2000 (2000-03-15), pages 659-669, XP002162237 figures 2,3,5; tables 1,2 -& DATABASE EMBL 'Online! EBI; ACC. NO.: AJ249977, 7 January 2000 (2000-01-07) CARLING : "Homo sapiens mRNA for AMP-activated protein kinase gamma 3 subunit (AMPK gamma 3 gene)" XP002162239 abstract	1-4, 11-17, 20,37
P,X	MILAN ET AL.: "A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle" SCIENCE, vol. 288, 19 May 2000 (2000-05-19), pages 1248-1251, XP002162238 figures 1,2; tables 1-3	1-33

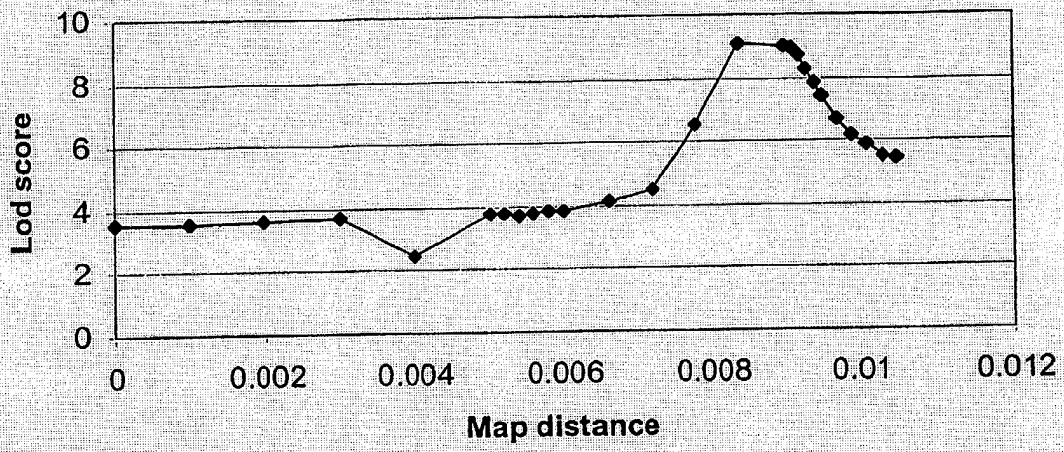
1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PC./EP 00/09896

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9725341 A	17-07-1997	AU 714905 B	13-01-2000
		AU 1693697 A	01-08-1997
		CA 2241786 A	17-07-1997
		EP 0873354 A	28-10-1998
		JP 2000503202 T	21-03-2000
		US 6124125 A	26-09-2000
WO 9858052 A	23-12-1998	US 5885803 A	23-03-1999
		AU 8154798 A	04-01-1999
		EP 1007692 A	14-06-2000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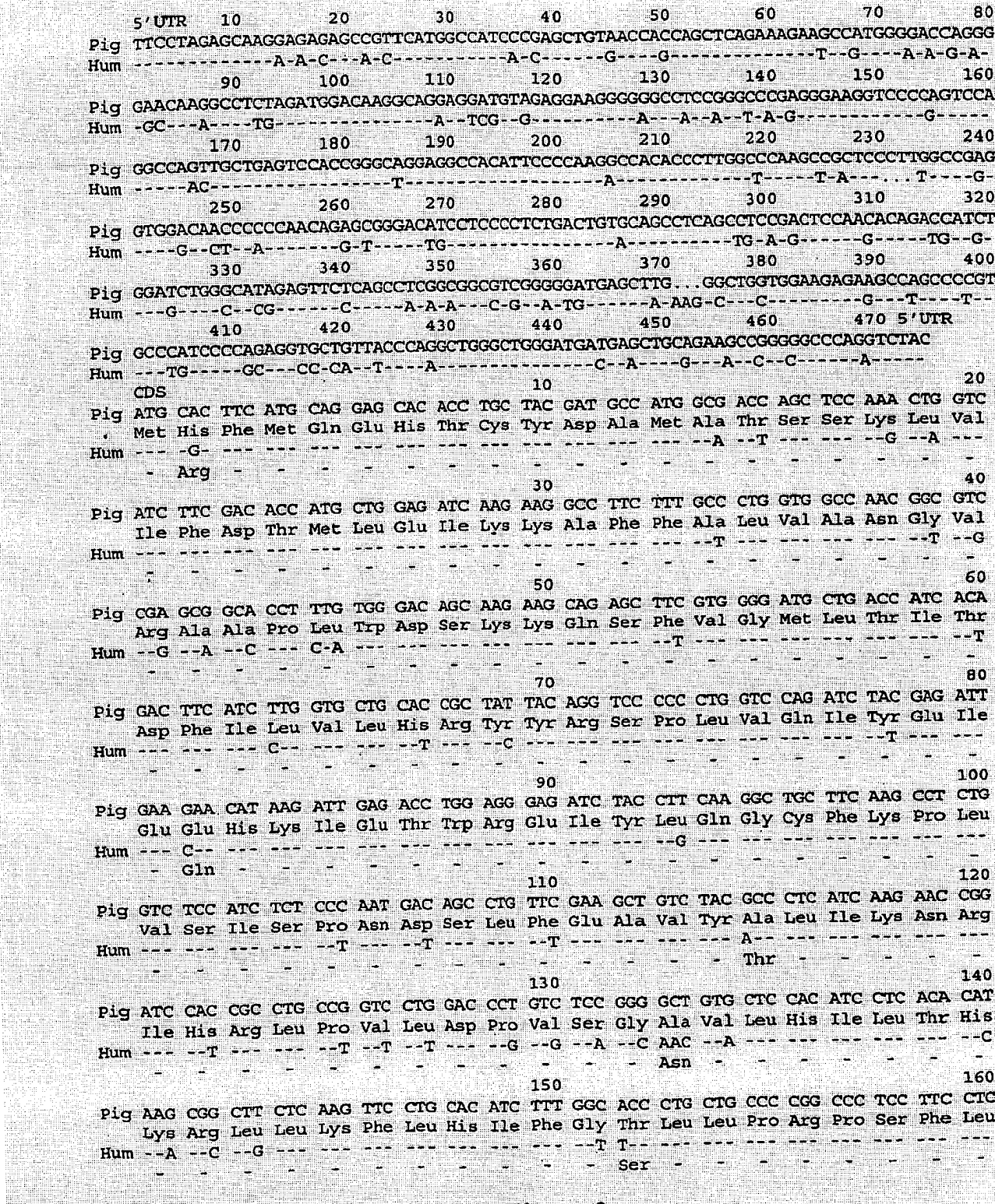
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Figure 2

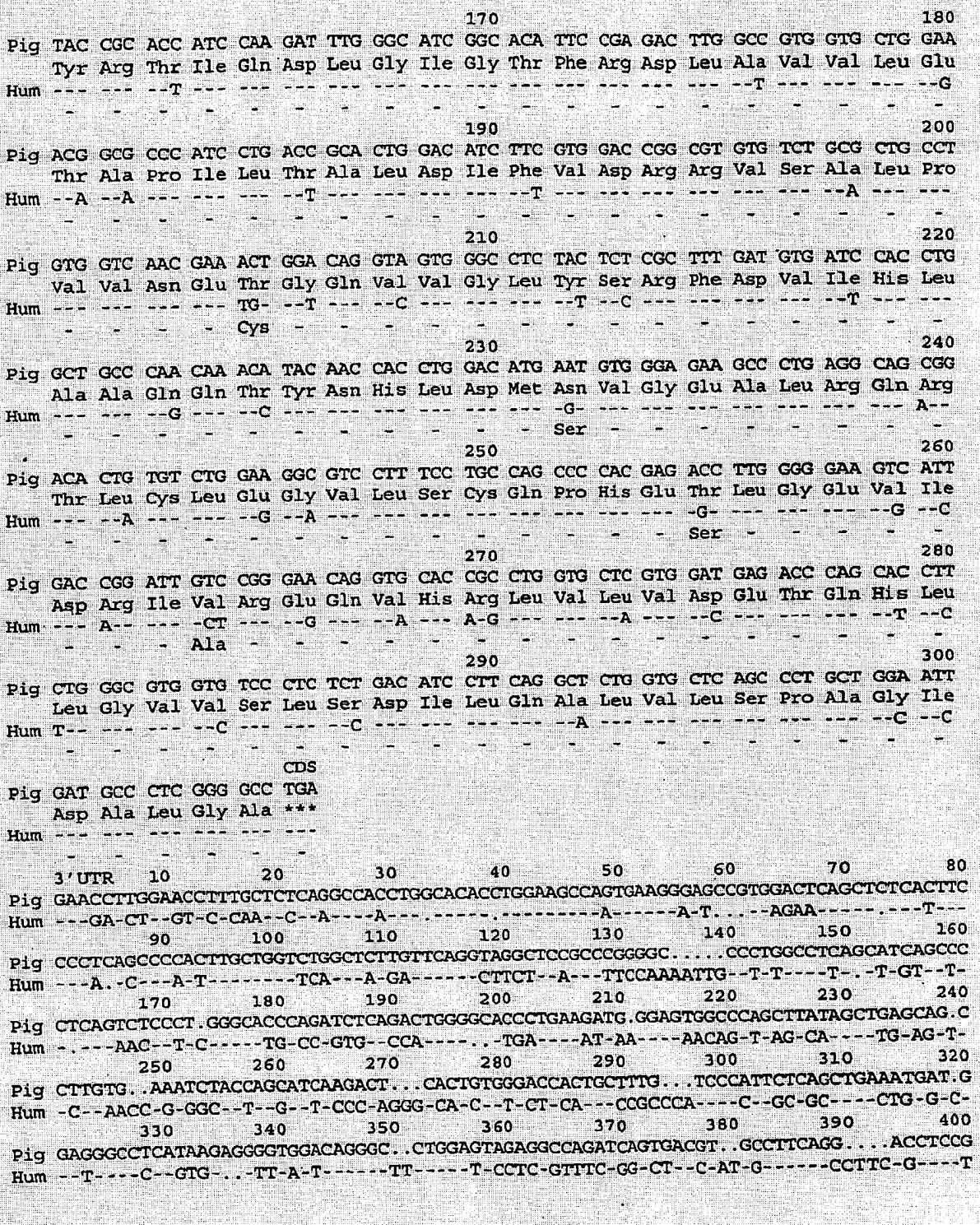

Figure 2 (cont.)

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Hum ------CCC-----TTG-...C---CAACGTCGC--C-G---T---A--CTCC-G-C-TTG-CATTTC---G-T-C-G-.
            490        500        510        520        530        540        550        560
Pig AAACCTTGGTTGGATGGAATTTCCACACTCG..........................................................
Hum --TG--GCA--TC-G--G....----CA-G-AGCAGCCGTTATTTATAGAACTGCCTGTTGGAGGTGGGGAGTCCTCCCT
            570        580        590        600        610        620        630        640
Pig ..........................................................................................
Hum CCATTCTTGTCCAGAAAACTCCTTAGCTCTCGCAGTGAGCCATGTTCTTAGTCTCCAGGGATGGATGGCCTTGTATATGG
            650        660        670        680        690        700        710        720
Pig ..........................................................................................
Hum ACCCCTGAGAATGAGCAATTGAGAAAACAAAACAAAAGGAACAATCCATGAACTTAGATTTTATTGGTTTCACTCAAAAT

            730        740
Pig ..................
Hum GCTGCAGTCATTTGACCTG
```

Figure 2 (cont.)

TABLE 2

	PigG3	HumG3	HumG1	RatG1	MusG1	HumG2	Dros	SNF4
PigG3	-	97.0	64.2	64.2	63.9	62.6	53.2	34.0
HumG3	90.7	-	63.6	63.6	63.6	62.6	53.5	34.4
HumG1	64.2	64.5	-	96.7	96.3	75.6	60.9	33.5
RatG1	65.8	65.8	88.0	-	97.4	75.3	61.1	33.5
MusG1	65.3	64.8	87.2	92.8	-	74.6	61.7	33.5
HumG2	61.6	61.6	68.1	67.8	65.9	-	63.1	34.5
Dros	58.4	58.4	59.0	59.3	59.0	60.0	-	36.2
SNF4	44.0	44.2	45.4	44.6	45.3	45.7	44.8	-

Figure 4 shows a Neighbor-Joining phylogenetic tree constructed with the PAUP software (SWOFFORD, *Phylogenetic analysis using parsimony (and other methods)*, Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts, 1998) using yeast SNF4 as outgroup; support for branch orders obtained in bootstrap analysis with 1,000 replicates are indicated, scales of tree is indicated at the bottom. The result showed that the pig gene located in the *RN* region is distinct from mammalian *PRKAG1* and *PRKAG2* isoforms and most likely orthologous to a human gene represented by the human EST sequence AA178898 (GenBank) derived from a muscle cDNA library. This gene is herein denoted *PRKAG3* since it is the third isoform of a mammalian AMP-activated protein kinase γ characterised so far.

The cDNA sequence of this gene was determined by RT-PCR and 5'RACE analysis using human skeletal muscle cDNA (Clontech, Palo Alto, CA). This sequence is shown in Figure 2 and in the sequence listing under SEQ ID NO: 3. The deduced polypeptidic sequence having 97% identity with the porcine sequence SEQ ID NO: 2 (cf. Table 2) is shown on Figure 2 and in the sequence listing under SEQ ID NO: 4.

The complete cDNA sequence is also shown in the enclosed sequence listing under SEQ ID NO: 29; the deduced polypeptidic sequence is shown in the enclosed sequence listing under SEQ ID NO: 30 and in Figure 3.

Using the high resolution human TNG radiation hybrid panel : (http://shgc-www.stanford.edu/RH/TNGindex.html) we mapped the human homologs of *PRKAG3*, *CYP27A1* and *KIAA0173*, all present in the porcine BAC127G6. The three genes are also very closely linked in the human genome. *PRKAG3* was mapped at a distance of 33 $cR_{50.000}$ from *KIAA0173* and 52 $cR_{50.000}$ from *CYP27A1*, with lod score support of 6.8 and 4.5, respectively.

The established role of AMPK in regulating energy metabolism, including glycogen storage, and its location in the region showing maximum linkage disequilibrium made *PRKAG3* a very strong candidate gene for *RN*. This was further strengthened by hybridisation analysis of a human multiple tissue northern blots (CLONTECH, Palo Alto, CA) using human *PRKAG1* (IMAGE clone 0362755 corresponding to GenBank entry AA018675), human *PRKAG2* (IMAGE clone 0322735 corresponding to GenBank entry W15439) and a porcine *PRKAG3* probe. The results are shown in Figure 5.

Legend of Figure 5:

H: Heart, B: Brain, Pl: Placenta, L: Lung, Li: Liver, M: Skeletal muscle, K: Kidney, Pa: Pancreas, S: Spleen, Th: Thymus, P: Prostate, T: Testis, O: Ovary, I: Small intestine, C: Colon (mucosal lining), PBL: Peripheral Blood Leukocyte.

While the *PRKAG1* and *PRKAG2* probes showed a broad tissue distribution of expression, *PRKAG3* showed a distinct muscle-specific expression. This result is also supported by the human EST database where multiple ESTs representing *PRKAG1* and *PRKAG2* have been identified in various cDNA libraries whereas a single EST (GenBank entry AA178898) representing *PRKAG3* has been obtained from a muscle cDNA library. The muscle-specific expression of *PRKAG3* and the lack of expression in liver are entirely consistent with the phenotypic effect of *RN*, namely that glycogen content is altered in muscle but

normal in liver (ESTRADE *et al.*, Comp. Biochem. Physiol. 104B, 321-326, 1993).

PRKAG3 sequences were determined from rn^+/rn^+ and RN^-/RN^- homozygotes by RT-PCR analysis. A comparison revealed a total of seven nucleotide differences four of which were nonsynonymous substitutions was found between the sequence from rn^+ and RN^- animals, as shown in Table 3 below. Screening of these seven SNPs with genomic DNA from additional rn^+ and RN^- pigs of different breeds revealed five different *PRKAG3* alleles, but only the R41Q missense substitution was exclusively associated with RN^-. This nonconservative substitution occurs in CBS1 which is the most conserved region among isotypic forms of the AMPK γ chain and arginine at this residue (number 70 in Prkag1) is conserved among different isoforms of mammalian AMPK γ sequences as well as in the corresponding *Drosophila* sequence (Figure 3). A simple diagnostic DNA test for the R41Q mutation was designed based on the oligonucleotide ligation assay (OLA; LANDEGREN *et al.*, Science, 241, 1077-1080, 1988). Screening a large number of RN^- and rn^+ animals from the Hampshire breed as well as large number of rn+ animals from other breeds showed that the 41Q allele was present in all RN^- animals but not found in any rn^+ animals, as shown in Table 4 below. The absence of the 41Q allele from other breeds is consistent with the assumption that the RN^- allele originated in the Hampshire breed; the allele has not yet been found in purebred animals from other breeds. In conclusion, the results provide convincing evidence that *PRKAG3* is identical to the *RN* gene and that the R41Q substitution most likely is the causative mutation.

able 3. Comparison of the *PRKAG3* sequences associated with the *rn*$^+$ and *RN* alleles in different pig populations[a]

Associated allele	*RN* allele	nt83	nt152	Codon 34	Codon 35	Codon 40	Codon 41	Codon 213	Population[b]
	RN	ACC	CTC	GCC	CTG	GTC	CAA	TCT	H
		T	L	A	L	V	Q	S	
	rn$^+$	---	---	---	---	---	-G-	---	L, LW, WB
		-	-	-	-	-	R	-	
	rn$^+$	---	-C-	--T	T--	---	-G-	--C	H, L, LW, M, WB
		-	P	-	-	-	R	-	
	rn$^+$	-A-	-C-	--T	T--	---	-G-	--C	D, H
		N	P	-	-	-	R	-	
	rn$^+$	---	-C-	--T	T--	A--	-G-	--C	H, LW, WB, D, L
		-	P	-	-	I	R	-	

ucleotide and codon numbers refer to the numbering of the sequence SEQ ID NO: 1
I=Hampshire, L=Landrace, LW=Large White, M=Meishan, WB=Wild Boar, D=Duroc
.D.=not determined, "-" indicates identity to the top sequence.

TABLE 4

RN phenotype	Genotype at nucleotide 593[d]			Total
	A/A	G/A	G/G	
RN$^-$, Hampshire[a]	40	87	0	127
RN$^-$, Hampshire[a,b]	0	13	0	13
rn$^+$, Hampshire[a]	0	0	60	60
rn$^+$, other breeds[c]	0	0	488	488

[a]represent both French and Swedish Hampshire populations
[b]heterozygosity *RN$^-$/rn$^+$* deduced using pedigree information
[c]breeds: Angler Saddleback, n=31; Blond Mangalitza, n=2; Bunte Bentheimer, n=16; Duroc, n=160; Göttinger Minipig, n=4; Landrace, n=83; Large White, n=72; Meishan, n=8; Piétrain, n=75; Red Mangalitza, n=5; Rotbunte Husumer, n=15; Schwalbenbauch Mangalitza, n=7; Schwäbisch Hällische, n=2; European Wild Boar, n=5; Japanese Wild Boar, n=3.
[d]**refers to the nucleotide numbers of SEQ ID NO: 1**

Without being bound to any particular mechanism, it may be hypothesised that the AMPK heterotrimer including PRKAG3 is involved in the regulation of glucose transport into skeletal muscle.

It has recently been reported that AMPK activation induced by the AMP analogue AICAR or by muscle contraction leads to an increased glucose uptake in skeletal muscle (BERGERON *et al.*, Am. J. Physiol., 276, E938-944, 1999; HAYASHI *et al.*, Diabetes, 47, 1369-1373, 1998). If this is the function of the AMPK heterotrimer including PRKAG3, R41Q may be a gain-of-function mutation causing a constitutively active holoenzyme, for instance due to the loss of an inactivating allosteric site. If so, the reduced AMPK activity in RN$^-$ animals is likely to reflect feed-back inhibition due to the high-energy status of the muscle. An increased uptake of glucose to skeletal muscle is expected to lead to an increase in muscle glycogen content as observed in RN$^-$ animals. It has been shown that overexpression of glucose transporter 4 (GLUT4) in transgenic mice leads to increased uptake of glucose and increased glycogen storage (TREADWAY *et al.*, J. Biol. Chem., 269, 29956-29961, 1994). This type of gain-of-function model is consistent with the dominance

of *RN*⁻ as the presence of a single unregulated copy would have a large effect on AMPK enzyme activity.

An alternative hypothesis on the functional significance of the R41Q substitution associated with the *RN*⁻ allele may also be proposed. Based on the established roles of the yeast SNF1 enzyme in utilisation of glycogen and of mammalian AMPK for inhibiting energy-consuming pathways and stimulating energy-producing pathways, activated AMPK is expected to inhibit glycogen synthesis and stimulate glycogen degradation. If this is the functional role of the isoform(s) containing the *PRKAG3* product, the R41Q substitution would be a loss-of-function mutation or a dominant-negative mutation locking the AMPK heterotrimer in an inactive state, and thus inhibiting AMP activation and glycogen degradation. In these cases the phenotypic effect should be explained by haplo-insufficiency, since *RN*⁻ appears fully dominant.

R41Q may thus be a dominant negative mutation, but only if it interferes with multiple isoforms since the major AMPK activity in muscle appears to be associated with the PRKAG1 and 2 isoforms [CHEUNG, et al. *Biochem. J.* 346, 659 (2000)].

The distinct phenotype of the *RN*⁻ mutation indicates that PRKAG3 plays a key role in the regulation of energy metabolism in skeletal muscle. For instance, PRKAG3 is likely to be involved in the adaptation to physical exercise, which is associated with increased glycogen storage. It is also conceivable that loss-of-function mutations in *PRKAG3* (or other AMPK genes) may predispose individuals to noninsulin-dependent diabetes mellitus, and AMPK isoforms are potential drug targets for treatment of this disorder.

EXAMPLE 2: DETECTION OF THE R41Q SUBSTITUTION IN PIG *PRKAG3*

A part of *PRKAG3* including codon 41 was amplified in 10 µl reactions containing 100 ng genomic

DNA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 4.0 pmol of both forward (AMPKG3F3:5'-GGAGCAAATGTGCAGACAAG-3') and reverse (AMPKG3R2:5'-CCCACGAAGCTCTGCTTCTT-3') primer, 10% DMSO, 1 U of *Taq* DNA polymerase and reaction buffer (ADVANCED BIOTECH, London, UK). The cycling conditions included an initial incubation at 94°C for 5 min followed by 3 cycles at 94°C (1 min), 57°C (1 min) and 72°C (1 min), and 35 cycles of 94°C (20 sec), 55°C (30 sec) and 72°C (30 sec). Allele discrimination at nucleotide position 122 was done using the oligonucleotide ligation assay (OLA, LANDEGREN *et al.*, Science, 241, 1077-1080, 1988). The OLA method was carried out as a gel-based assay. Each 10 µl OLA reaction contained 0.5 pmol of each probe SNPRN-A (5'Hex-TGGCCAACGGCGTCCA-3'), SNPRN-G (5'ROX-GGCCAACGGCGTCCG-3') and SNPRN-Common (5'phosphate-AGCGGCACCTTTGTGAAAAAAAAAA-3'), 1.5 U of thermostable AMPLIGASE and reaction buffer (EPICENTRE TECHNOLOGIES, Madison, WI) and 0.5 µl of the AMPKG3F3/AMPKG3R2 PCR product. After an initial incubation at 95°C for 5 min, the following thermocycling profile was repeated 10 times: denaturation at 94°C (30 sec), and probe annealing and ligation at 55°C (90 sec). After OLA cycling, 1 µl of product was heat denatured at 94°C (3 min), cooled on ice, and loaded onto 6% polyacrylamide denaturing gel for electrophoresis on an ABI377 DNA sequencer (PERKIN ELMER, Foster City, USA). The resulting fragment lengths and peak fluorescence were analysed using GENESCAN software (PERKIN ELMER, Foster City, USA).

The OLA-based method for the R41Q mutation was used to determine the genotype of DNA samples collected from 68 Swedish Hampshire animals phenotyped as either RN^- or rn^+ based on their glycolytic potential (GP) value. Figure 6 illustrates typical OLA results from the three possible genotypes. All RN^- animals were scored as homozygous A/A (n=28) or heterozygous A/G (n=36) at

nucleotide position 122 whereas the rn^+ animals were homozygous G/G (n=4) at this position.

EXAMPLE 3: PREDICTING THE PRESENCE OF THE *RN*⁻ ALLELE USING A CLOSELY LINKED MICROSATELLITE, MS127B1

A microsatellite 127B1 (*MS127B1*) was cloned from BAC 127G7 containing pig *PRKAG3*. The BAC clone was digested with *Sau*3AI and the restriction fragments subcloned into the *Bam*HI site of pUC18. The resulting library was probed with a $(CA)_{15}$ oligonucleotide probe labelled with [γ-32P]-dATP. Strongly hybridising clones were sequenced and primers for PCR amplification of microsatellite loci were designed. Ten µl PCR reactions were performed containing 100 ng genomic DNA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 4.0 pmol of both forward (MS127B1F:5'-Fluorescein-CAAACTCTTCTAGGCGTGT-3') and reverse (MS127B1R:5'-GTTTCTGGAACTTCCATATGCCATGG-3') primers, and 1 U of *Taq* DNA polymerase and reaction buffer (ADVANCED BIOTECH, London, UK). The cycling conditions included an initial incubation at 94°C for 5 min followed by 3 cycles at 94°C (1 min), 57°C (1 min) and 72°C (1 min), and 35 cycles of 94°C (20 sec), 55°C (30 sec) and 72°C (30 sec). The PCR products (0.3 µl) were separated using 4% polyacrylamide denaturing gel electrophoresis on an ABI377 DNA sequencer (PERKIN ELMER, Foster City, USA). The resulting fragment lengths were analysed using the GENESCAN and GENOTYPER software (PERKIN ELMER, Foster City, USA).

The method was used to determine the genotype of DNA samples collected from 87 Swedish Hampshire animals phenotyped as either RN^- or rn^+ based on their glycolytic potential (GP) value. Allele 108 (bp) showed a complete association to the *RN*⁻ allele in this material as all RN^- (*RN*⁻/*RN*⁻ or *RN*⁻/*rn*⁺) animals were homozygous or heterozygous for this allele while no rn^+ (*rn*⁺/*rn*⁺) animals carried this allele, as shown in Table 5 below.

TABLE 5

Animals	n	Genotype				
		94/94	94/108	94/114	100/108	108/108
RN^-	80	0	37	0	2	41
rn^+	7	3	0	4	0	0

EXAMPLE 4: DETECTING THE PRESENCE OF THE *RN*⁻ ALLELE USING A PCR-RFLP TEST

The *RN*⁻ mutation inactivates a *Bsr*BI site GAG^CGG/CTC^GCC (*Bsr*BI RE site is not palindromic). At that site, the *RN*⁻ sequence is AAGCGG instead of GAGCGG.

A 134 bp long fragment of the *RN* gene is amplified from porcine genomic DNA. The *rn*⁺ allele is identified after *Bsr*BI digestion, by detection of two fragments of 83 and 51 bps.

The test is performed as follows:

1° Primer sequences:

Sequence of primers used to amplify the *RN* mutation region:

RNU: 5' GGGAACGATTCACCCTCAAC 3'

RNL: 5' AGCCCCTCCTCACCCACGAA 3'

To provide an internal control of digestion, a *Bsr*BI site has been added at the extremity of one of the two primers within a 20 bp long tail. The tail permits both creation of a *Bsr*BI site (a shorter tail might be sufficient), and an easy discrimination of uncut fragment from other fragments. The use of tailed primers does not affect efficiency and specificity of amplification.

The sequence of the RNL modified primer including a control tail with a *Bsr*BI site is:

RNLBsrA14: 5'

$A_5C_2A_7$CCGCTCAGCCCCTCCTCACCCACGAA 3'

2° PCR reaction mixture used:

50 ng DNA

0.5 Unit *Taq* polymerase (GIBCO BRL)

1.5 mM $MgCl^2$

200 mM dNTP

0.2 µM each primer
Total reaction volume: 25 µl

3° PCR conditions used (on OMNIGENE HYBAID thermocycler):

1x (5min 95°C)
35x (45sec 57°C, 45sec72°C, 45sec95°C)
1x (45sec57°C, 15min 72°C)

4° Restriction enzyme digestion performed at 37°C for 2 hours:

10 µl PCR product
1x *Bsr*BI BIOLABS buffer
5U *Bsr*BI restriction enzyme (BIOLABS)
Total reaction volume: 15 µl

5° Size of fragments produced after PCR using primers with control tail and digestion with *Bsr*BI:

Uncut fragment from *RN*⁻ or *rn*⁺ allele : 154 bp
After digestion of fragment amplified from *RN*⁻ allele : 137 bp + 17 bp
After digestion of fragment amplified from *rn*⁺ allele : 83 bp + 54 bp + 17 bp

Size difference can be identified either after polyacrylamide, agarose/NUSIEVE or agarose gel electrophoresis.

EXAMPLE 5: EFFECT OF V40I POLYMORPHISM ON GLYCOLYTIC POTENTIAL.

Further, a set of 181 *rn*⁺/*rn*⁺ homozygous animals (R/R at position 41 of SEQ ID NO: 2) were analyzed for the V40I polymorphism (referring to position 40 of SEQ ID NO: 2) by PCR-RFLP using *Fok*I restriction enzyme. The glycolytic potential was determined in parallel according to the method disclosed by MONIN *et al.*, (Meat Science, 13, 49-63, 1985).

The results are shown in Table 6 below:

Table 6

Genotype at position 40	Average glycolytic potential	Standard Deviation	Number of typed animals
I/I	178.30	31.13	13
V/I	204.15	37.73	164
V/V	210.83	38.21	104

These results show that the V40I polymorphism has a significant effect on the glycolytic potential in skeletal muscle.

CLAIMS

1) A gamma subunit of a vertebrate AMP-activated kinase (AMPK), wherein said gamma subunit is a polypeptide comprising at least a sequence having at least 70% identity with the polypeptide SEQ ID NO: 2.

2) A polypeptide of claim 1, wherein said polypeptide comprises a sequence having at least 95% identity with the polypeptide SEQ ID NO:2.

3) A polypeptide of claim 1, wherein said polypeptide comprises a sequence having at least 75% identity with the polypeptide SEQ ID NO: 28.

4) A polypeptide of any of claims 1 to 3, wherein said polypeptide comprises the sequence SEQ ID NO: 2 or SEQ ID NO:4.

5) A polypeptide of claim 4, wherein said polypeptide comprises the sequence SEQ ID NO: 28, SEQ ID NO: 30 or SEQ ID NO: 32.

6) A polypeptide which is a functionally altered mutant of a gamma subunit of a vertebrate AMP-activated kinase, wherein said polypeptide has at least a mutation located within the first CBS domain of said gamma subunit.

7) A polypeptide of claim 6, wherein the mutation is located within the region of the first CBS domain aligned with the region of a polypeptide of SEQ ID NO: 2 spanning from residue 30 to residue 50.

8) A polypeptide of claim 7, wherein the mutation is a R→Q substitution or a V→I substitution.

9) A polypeptide of claim 8 selected among:

- a polypeptide having a sequence resulting from a R→Q substitution at a position corresponding to position 41 in SEQ ID NO: 2;

- a polypeptide having a sequence resulting from a V→I substitution at the position corresponding to position 40 of SEQ ID NO: 2.

10) A polypeptide which is a mutant of a gamma subunit of a vertebrate AMP-activated kinase, wherein said polypeptide results from a deletion of a part of a polypeptide of any of claims 1 to 5.

11) A nucleic acid sequence encoding a polypeptide of any of claims 1 to 10, or the complement thereof, provided that said nucleic acid sequence does not consist of the EST GENBANK AA178898, or of the EST W94830.

12) A nucleic acid sequence of claim 11, having the sequence SEQ ID NO: 1, SEQ ID NO:3, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, or the complement thereof.

13) A nucleic acid sequence comprising at least a portion of a nucleic acid sequence encoding a polypeptide of any of claims 1 to 10, and up to 500 kb of a 3′ and/or of a 5′ adjacent genomic DNA sequence, or the complement thereof.

14) A nucleic acid fragment selected among:

- a specific fragment of a nucleic acid sequence encoding a polypeptide of any of claims 1 to 10, or of a nucleic acid sequence of claim 13;
- a nucleic acid fragment which specifically hybridises under stringent conditions with a nucleic acid sequence encoding a polypeptide of any of claims 1 to 8, or of a nucleic acid sequence of claim 11;

provided that said nucleic acid fragment does not consist of the EST GENBANK AA178898 or of the EST GENBANK W94830.

15) A set of primers for amplifying a nucleic acid sequence of any of claims 11 to 13 or a portion thereof, comprising at least a primer consisting of a nucleic acid fragment of claim 14.

16) A recombinant vector comprising a nucleic acid sequence encoding a polypeptide of any of claims 1 to 10.

17) An host cell transformed by a nucleic acid sequence encoding a polypeptide of any of claims 1 to 10.

18) A transgenic animal transformed by a nucleic acid sequence encoding a polypeptide of any of claims 1 to 10.

19) A knockout animal, wherein the gene encoding a polypeptide of any of claims 1 to 5 is inactive.

20) A heterotrimeric AMPK wherein the γ subunit consists of a polypeptide of any of claims 1 to 10.

21) A method of detecting a metabolic disorder resulting from a mutation in a gene encoding a γ subunit of AMPK, wherein said process comprises:

- obtaining a nucleic acid sample from a vertebrate;
- checking the presence in said nucleic acid of a nucleic acid sequence encoding a polypeptide of any of claims 1 to 10, wherein said polypeptide is functionally altered.

22) A method of claim 21 wherein the disorder is correlated with an altered glycogen accumulation in the muscular cells and results from the expression of a functionally altered allele of a polypeptide of any of claims 1 to 5.

23) A method of any of claims 21 or 22 wherein the presence of the nucleic acid sequence encoding said mutant polypeptide is checked by contacting said nucleic acid sample with a nucleic acid probe obtained from a nucleic acid of claim 14 and spanning said mutation, under conditions of specific hybridisation between said probe and the mutant sequence to be detected, and detecting the hybridisation complex.

24) A method for obtaining a pair of primers allowing to detect a genetic polymorphic marker linked to

a nucleic acid sequence encoding a polypeptide of any of claims 1 to 5, wherein said process comprises:

- screening a genomic DNA library from a vertebrate with a probe specific for a nucleic acid sequence encoding a polypeptide of any of claims 1 to 5, in order to select clones comprising said nucleic acid sequence and flanking chromosomal sequences;
- identifying a polymorphic locus in said flanking chromosomal sequences, and sequencing a DNA segment comprising said polymorphic locus ;
- designing primer pairs flanking said polymorphic locus.

25) A method of claim 24 wherein the selected clones comprise at least a portion of a nucleic acid sequence encoding a polypeptide of any of claims 1 to 5, and up to 500 kb of a 3' and/or of a 5' adjacent sequence.

26) A method of any of claims 21 to 25 wherein the vertebrate is a mammal.

27) A method of claim 26 wherein said mammal is a pig.

28) A pair of primers obtainable by the process of any of claims 24 to 26.

29) A process for detecting a dysfunction of carbohydrate metabolism resulting from the expression of a functionally altered allele of a polypeptide of any of claims 1 to 5 in a vertebrate, wherein said process comprises:

- obtaining a sample of genomic DNA from said vertebrate;
- contacting said DNA with a pair of primers of claim 28 under conditions allowing PCR amplification;
- analysing the PCR product to detect if an allele of a polymorphic marker linked to a nucleic acid sequence encoding a functionally altered allele of a polypeptide of any of claims 1 to 5 is present.

30) A process of claim 29, wherein said functionally altered polypeptide results from a R41Q substitution in SEQ ID NO: 2.

31) A process of any of claims 29 or 30, wherein said vertebrate is a mammal.

32) A process of claim 31 wherein said mammal is a pig.

33) A process of claim 32 wherein the pair of primers is selected among:

- a pair of primers consisting of SEQ ID NO: 5 and SEQ ID NO: 6;
- a pair of primers consisting of SEQ ID NO: 7 and SEQ ID NO: 8;
- a pair of primers consisting of SEQ ID NO: 9 and SEQ ID NO: 10;
- a pair of primers consisting of SEQ ID NO: 11 and SEQ ID NO: 12;
- a pair of primers consisting of SEQ ID NO: 13 and SEQ ID NO: 14;
- a pair of primers consisting of SEQ ID NO: 15 and SEQ ID NO: 16;
- a pair of primers consisting of SEQ ID NO: 17 and SEQ ID NO: 18;
- a pair of primers consisting of SEQ ID NO: 19 and SEQ ID NO: 20;
- a pair of primers consisting of SEQ ID NO: 21 and SEQ ID NO: 22;
- a pair of primers consisting of SEQ ID NO: 23 and SEQ ID NO: 24;
- a pair of primers consisting of SEQ ID NO: 25 and SEQ ID NO: 26.

34) Use of a transformed cell of claim 17 to screen compounds able to modulate AMPK activity.

35) Use of a transgenic animal of claim 18 to screen compounds able to modulate AMPK activity.

36) Use of a knockout animal of claim 19 to screen compounds able to modulate energy metabolism in the absence of a functional polypeptide of any of claims 1 to 5.

37) Use of an heterotrimeric AMPK of claim 20 to screen compounds able to modulate AMPK activity.